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Protective effect of acetic acid against ethanol-induced cell death in *Saccharomyces cerevisiae*

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Trabalho efectuado sob orientação da
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Efeito protetor do ácido acético na morte celular induzida por etanol em Saccharomyces cerevisiae

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Resumo

O etanol é um produto final bem conhecido da fermentação alcoólica realizada por *Saccharomyces cerevisiae*. Em altas concentrações, é responsável pela redução de viabilidade celular e inibição da fermentação. Além disso, durante a fermentação alguns ácidos fracos, como os ácidos acético, butírico e pirúvico, produzidos pelo metabolismo da levedura, podem acumular-se no meio de crescimento e aumentar a toxicidade do etanol, o que resulta numa maior inibição de crescimento e fermentação (Gibson, *et al*., 2007). No entanto, dados obtidos anteriormente mostraram que culturas celulares de *S. cerevisiae* tratadas simultaneamente com concentrações tóxicas de etanol e baixas concentrações de ácido acético exibiam maiores taxas de sobrevivência (medido pela contagem de UFC e por ensaios citométricos) quando comparado com células tratadas apenas com etanol. Estes resultados indicam que o ácido acético induz uma resposta celular que confere proteção contra os efeitos citotóxicos do etanol (Vieira *et al.*, resultados não publicados).

Posteriormente, foi demonstrado que a MAP cinase Hog1p e a aquogliceroporina Fps1p, envolvidas na resistência em ácido acético, não mediam este efeito protetor do ácido (Trindade, 2009).

O objetivo do presente trabalho foi estudar o papel de outras vias de sinalização e os mecanismos envolvidos na proteção do ácido acético contra a morte induzida pelo etanol (13% v / v, pH 3,5). Demonstramos que esse efeito depende da dose de ácido adicionado, sendo a concentração ótima 0,1% (v / v). Ácido propiónico, à semelhança do ácido acético e contrariamente ao ácido lático e fórmico, também confere proteção. O mutante na cinase Slt2/Mpk1, conhecida por ser ativada na presença de ácido acético (Mollapour e Piper, 2006), reverte parcialmente o efeito de proteção do ácido, especialmente em relação à preservação da integridade da membrana plasmática. A presença de trealose no meio de cultura promove o efeito protetor do ácido acético. O transporte e acumulação de trealose parecem ser necessários para a proteção por ácido acético contra a perda da integridade da membrana plasmática, mas não de viabilidade celular induzida por etanol. Culturas celulares com a cadeia respiratória afectada (ρ^0) não apresentam maiores taxas de viabilidade célular na presença de ácido acético, quando comparado com as mesmas culturas tratadas apenas com etanol, sugerindo que a mitocôndria pode estar envolvida no efeito protetor.

Abstract

Ethanol is a well-known end product of alcoholic fermentation carried out by *Saccharomyces cerevisiae*. At high concentrations it reduces cell viability and inhibits fermentation. During fermentation, some weak acids such as acetic, butyric and pyruvic acids, produced by yeast metabolism, may accumulate in the growth medium and enhance ethanol toxicity, which results in a higher inhibition of yeast growth and of fermentation (Gibson, *et al*., 2007) However, previous data obtained in our lab showed that *S. cerevisiae* cells exposed simultaneously to toxic concentrations of ethanol and low concentrations of acetic acid displayed higher survival (measured either by CFU or by propidium iodide staining) than cells treated only with ethanol. These results indicated that acetic acid induces a cellular response that provides protection against the cytotoxic effect of ethanol (Vieira *et al.*, unpublished results). Subsequently, it was shown that the Mitogen-Activated Protein Kinase (MAPK) Hog1p and the aquoglyceroporin Fps1p, involved in acetic acid resistance, did not mediate this protective effect of the acid (Trindade, 2009).

The aim of the present work was to study the role of other signaling pathways and the mechanisms by which acetic acid confers protection against death induced by ethanol (13% v/v, pH 3.5). We found that this effect is dose-dependent and optimal for 0.1% (v/v) of acetic acid. Propionic, but not lactic or formic acids, also confer protection from ethanol-induced cell death. Deficiency in the MAPK Slt2/Mpk1, which is activated in the presence of acetic acid (Mollapour and Piper, 2006), partially reverts the acetic acid protective effect, especially in the preservation of plasma membrane integrity. We also found that the presence of trehalose in the culture medium promoted the protective effect of acetic acid. Trehalose transport and accumulation seem to be necessary for acetic acid to protect cells from loss of the plasma membrane integrity induced by ethanol, but not of cell viability. Finally, we show that the protective effect of acetic acid was not observed in a respiratory deficient mutant (ρ^0), suggesting that mitochondria may be involved in this process.

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Abbreviations

CDI	Cyclin-Dependent kinase Inhibitors
C.F.U.	Colony Forming Units
DNA	Deoxyribonucleic acid
ESR	Environmental Stress Response
Fis1	Mitochondrial fission protein
GSSG	Glutathione disulfide
GFP	Green Fluorescence Protein
HSEs	Heat Shock Elements
HSP	Heat Shock Protein
LEA	Late Embryogenic Abundant
MAPK	Mitogen-activated protein kinase
MnSOD	Mitochondrial Manganese-Superoxide Dismutase
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
pH	Potential of Hydrogen
PHD	Plant Homeo Domain
PKC	Protein kinase C
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
TPS1	Trehalose-6-Phosphate Synthase
YPD	Yeast Peptone Dextrose
Wt	Wild-type
µL	Microliter

1. Introduction

1.1 Yeast: a model for fundamental and applied research

Yeasts are eukaryotic unicellular fungi that have ultrastructural features similar to those of higher eukaryotic cells. Moreover, they are easy to manipulate genetically and their growth/divison can be efficiently controlled, making yeast an advantageous eukaryote model for molecular and cellular biology studies.

The most commercially exploited yeast species, *Saccharomyces cerevisiae*, has been traditionally used in the brewing, baking and wine-making industries. With advances in yeast biotechnology, this organism became very important in different industries including pharmaceutical, food and beverages, chemical and in the agriculture and environment fields.

Current pressure to find renewable fuel alternatives increased the interest in biotechnological applications of yeast to produce a gasoline substitute such as bioethanol. Bioethanol is the biofuel with the highest production worldwide and several strategies are being developed to enhance its production on a large scale (Muthaiyan and Ricke, 2009). The first biological process used for producing ethanol at an industrial scale was the fermentation of sugars by yeasts. *S. cerevisiae* is an example of an organism used in biofuel bioprocessing, but other species are also used such as other *Saccharomyces* spp, *Kluyveromyces marxianus*, *Pachysolen tannophilus* and *Sheffersomyces stipitis* (Muthaiyan and Ricke, 2009).

In agriculture, several yeast species have proven beneficial to plants, preventing fungal disease. More benefits of yeasts were found in certain aspects of pollution control; for instance, yeast can absorb heavy metals and detoxify chemical pollutants from industrial effluents (Madeo, *et al*., 2002).

Although yeasts have a lot of applications in several sectors, one of the most important is their use as a model for medical research, such as in the understanding of eukaryotic cell division, cell death and human pathologies (Madeo, *et al*., 2002). The presence of an apoptosis regulatory network in yeast that encompasses many of the crucial events that occur in mammalian cells stimulated several studies in disease-related proteins that have no homologues in this organism and created a new research

field with the so-called humanized yeast systems, providing cell-based assays to discover novel medicinal compounds (Mager and Winderickx, 2005). Yeast recombinant DNA technology also provides a valuable tool to uncover potential novel human therapeutic agents. Yeasts have thus been an extremely valuable experimental model in biomedical research such as in oncology, pharmacology, toxicology, virology, and human genetics (see Table 1).

Tabel 1- Value of yeasts in biomedical research (Adapted from Walker, 2009)

Biomedical field	Examples
Oncology	Basis of cell cycle control, human oncogene (e.g., Ras) regulation; telomere function, tumor suppressor function, and design of (cyclin-dependent kinase inhibitors) CDIs/anti-cancer drugs
Aging and Apoptosis	Mechanisms of cell aging, longevity genes, and apoptosis
Pharmacology	Multidrug resistance, drug action/metabolism, and drug screening assays
Virology	Viral gene expression, antiviral vaccines, and prion structure/function
Human genetics	Basis of human hereditary disorders and genome/proteome projects

1.2 Stress in yeast

In their natural habitat, yeasts might be exposed to changes in environmental conditions, and extreme changes represent a stress to the cells. Stress-inducing conditions can be physical or chemical, such as changes in temperature, osmotic pressure, pH, concentration of water, ions and solutes, exposure to extremes of radiation, pressure, toxic chemicals or oxidative conditions and nutrient starvation. Stress can also be imposed by the host defense mechanism on pathogenic yeasts. An important host defense mechanism is the oxidative burst (production of high levels of reactive oxygen species - ROS) promoted by neutrophil cells used to eliminate pathogenic yeasts (Moye-Rowley, 2003).

Yeasts might be exposed to several stresses simultaneously or sequentially. When *Saccharomyces cerevisiae* cells are grown under fermentative conditions, they are initially exposed to a high sugar concentration that promotes osmotic stress. The sugar is then fermented to ethanol and the osmotic pressure is reduced, but simultaneously the concentration of ethanol increases and adds extra water and ethanol stress to yeast. Upon exposure to different stresses, yeast sequentially expresses different stress response genes (Zuzuarregui and del Olmo, 2004).

1.2.1 Ethanol stress

Ethanol is well known as the main stress factor during the process of alcoholic fermentation. At low concentrations, it is responsible for the inhibition of growth and cell division, and promotes a decrease in cell volume and specific growth rate. High concentrations of ethanol reduce the cell viability and increase cell death (Birch and Walker, 2000; Stanley, *et al*., 2010). Moreover, there are changes in metabolism such as decreased mRNA and protein levels, a decrease of different transport systems like the general amino acid permease system and glucose uptake, enhanced frequency of petite mutations, and inhibition of the activity of some glycolytic enzymes (Alexandre, *et al*., 2001). Exposing the cell to toxic levels of ethanol also promotes damage of mitochondrial DNA (You, *et al*., 2003), induction of heat shock proteins and other stress response proteins, as well as intracellular accumulation of trehalose.

Additional targets of ethanol are the cell membrane, hydrophobic and hydrophilic proteins, and the endoplasmatic reticulum (Walker and Maynard, 1997), although the structure and function of the cell membrane seems to be the predominant target. The effect of ethanol on cell structure and membrane function is accompanied by changes in vacuole morphology, inhibition of endocytosis, increased unsaturated ⁄saturated fatty acid ratio and ergosterol content in membranes, loss of electrochemical gradients and proton-motive force and an increase in membrane fluidity. These alterations in the fluidity of the plasma membrane promote changes in the permeability to ionic species, especially protons (Baker and Lynen, 1971). The increased proton influx across the plasma membrane causes intracellular acidification, which can be surpassed by the incorporation of oleic acid into lipid membranes that counteracts the fluidizing effects

of ethanol (Araki, *et al*., 2009; Casal, *et al*., 1998). In *S. cerevisiae*, ethanol stress can stimulate the Ca^{2+}-mediated calcineurin/Crz1 pathway (Das and Vasudevan, 2007). Ethanol is also known to promote oxidative stress by the production of ROS and RNS (Viegas and Sa-Correia, 1997). The main effects of ethanol on the yeast cell are summarized in Table 2. However, yeast has developed several stress response pathways that allow it to survive under stress conditions, such as changes in cell cycle progression, metabolic activity, and gene expression (Hohmann and Mager, 2003).

Table 2- Cell functions affected by ethanol (adapted from Stanley, *et al*., 2010)

Cell function and ethanol influence	**Source**
Cell viability and growth	
Inhibition of growth, cell division and cell viability	(Stanley, *et al*., 1997)
Decrease in cell volume	(Birch and Walker, 2000)
Metabolism	
Lowered mRNA and protein levels	(Chandler, 2004) (Hu, *et al*., 2007)
Protein denaturation and reduced glycolytic enzyme activity	(Hallsworth, 1998)
Induction of heat shock proteins and other stress response proteins	(Plesset, *et al*., 1982)
Intracellular trehalose accumulation	(Lucero, *et al*., 2000)
Cell structure and membrane function	
Altered vacuole morphology	(Meaden, *et al*., 1999)
Inhibition of endocytosis	(Lucero, *et al*., 2000)
Increased unsaturated/saturated fatty acid ratio in membranes	(Alexandre, *et al*., 1994a)
Increase in ergosterol content of membranes	(Sajbidor, *et al*., 1995)
Loss of electrochemical gradients and proton-motive force	(Petrov, 1990)
Inhibition of transport processes	(Leao, 1984)
Inhibition of H^+-ATPase activity	(Cartwright, 1986)
Increased membrane fluidity	(Mishra, 1989)

1.2.2 Weak carboxylic acids stress

It is well known that weak carboxylic acids control microbial growth, and they are used as preservatives in several industries such as the food, agriculture and pharmaceutical industries. Due to their reduced negative impact on the environment, they are also used as raw materials for several products, ranging from new plastics to pharmaceuticals and cosmetic products (Abbott, *et al*., 2009). *S. cerevisiae* has also proven to be an invaluable eukaryote model to study the cytotoxic effects and the cellular responses to weak carboxylic acids used as pharmaceuticals or pesticides (Mira, *et al*., 2010).

During fermentation, some weak carboxylic acids such as acetic, butyric and pyruvic acids, produced by yeast metabolism, accumulate in the growth medium. In conjunction with ethanol toxicity, this can result in growth arrest of fermenting cells and decrease the productivity of grape must fermentation (Gibson, *et al*., 2007).

The cytotoxic effect of acetic acid on the fermentative yeast *S. cerevisiae* and its role as a physiological inducer of apoptosis was recently reviewed (Vilela-Moura, *et al*., 2011). At low pH, the undissociated form of the weak acid predominates and may permeate the plasma membrane by simple diffusion (Casal, *et al*., 1996), Recently, it has been suggested that the aquaglyceroporin Fps1p can also be involved in acetic acid transport into the cell (Mollapour and Piper, 2007). Once inside the cell, where the pH is usually close to neutrality, the undissociated form of the acid can release its proton, promoting intracellular acidification, anion accumulation, and inhibition of cellular metabolic activity, namely fermentation (Pampulha and Loureiro-Dias, 1989) and alterations in glycolysis (Pampulha and Loureiro-Dias, 1990). Under certain conditions, acetic acid is also responsible for two types of cell death, high- and low-enthalpy (Pinto, *et al*., 1989). Assessment of cellular structural and functional changes pointed to an intracellular localization of the acetic acid cellular target(s) (Ludovico, 1999 ; Prudêncio, *et al*., 1998). The use of cellular death markers allowed the characterization of the cell death process. Exponential-phase cultures of *S. cerevisiae* treated with high concentrations of acetic acid (120-200 mM), exhibited a necrotic phenotype, while cultures treated with low doses (20-80 mM) evidenced a programmed cell death (PCD) process (Ludovico, *et al*., 2001). Cells exposed to low concentrations of acid displayed cycloheximide-inhibitable alterations in chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the surface of the cytoplasmic membrane and the occurrence

of DNA strand breaks. High levels of acid promote DNA breakdown into fragments of several hundred kilobases (Ribeiro, *et al*., 2006). There is also evidence that the acetic acid-induced PCD process in *S. cerevisiae* cells is accompanied by reactive oxygen species (ROS) accumulation, decrease in cytochrome oxidase activity affecting mitochondrial respiration, and other events that show the involvement of mithocondria in the yeast PCD process (Giannattasio, *et al*., 2005; Ludovico, *et al*., 2002).

Although weak acids inhibit cell growth, yeasts have developed adaptation mechanisms that allow them to grow in their presence, at low pH. When cells are exposed to inhibitory concentrations of acid in the growth medium, there is a growth arrest and cells initiate a latency period. However, when these cells are reinoculated into fresh-growth-medium under the same conditions of pH and acid content, no cell arrest is observed because cells had developed several adaptation molecular responses, which allow them to survive (Cabral, *et al*., 2001; Viegas and Sa-Correia, 1997).

1.3 Protection mechanisms in response to cell stress

Yeasts have developed mechanisms that enable them to survive under stress conditions and render the yeast used for baking, brewing and winemaking more tolerant to extreme conditions. These mechanisms are highly complex and tolerance is acquired through rapid molecular responses that protect against damage caused by ongoing exposure to the same or other agents of stress.

The cellular damage caused by one type of stress can be specific for this stress or common to other types of stress, and the mechanisms of protection and repair can thus partially overlap (Beck, *et al*., 2000; Davidson, *et al*., 1996; Mager, *et al*., 2000; Pahlman, *et al*., 2001; Rep, *et al*., 2000; Tanghe, *et al*., 2002). These responses include changes in gene transcription and translational and post-translational modifications of stress-associated proteins, and are triggered, at least in part, by stress-induced denaturation of proteins, disordering of membranes, DNA damage and metabolic disturbances (Mager and Ferreira, 1993; Piper, 1993; Siderius, *et al*., 1997). The major yeast stress-response mechanisms that protect yeast cells against various types of stress are: accumulation of trehalose, synthesis of molecular chaperones, antioxidant proteins and hydrophylins, accumulation of compatible solutes, and changes in the composition

of the plasma membrane. A specific mechanism to protect cells from freeze stress through the expression of aquaporins has also been described (Tanghe, *et al*., 2002).

1.3.1 Cell stress responses- protection mechanisms- trehalose accumulation

Trehalose (α-D-glucopyranosyl (1–1)-α-D-glucopyranoside) is a disaccharide produced by a wide variety of organisms. It is synthesized by a multimeric protein complex composed of Tps1p, Tls1p, Tps3p and Tps2p (Bell, *et al*., 1998; Reinders, *et al*., 1997). Tps1p is the only component that catalyzes the formation of trehalose from UDP-Glc and glucose-6-phosphate, and it is essential for growth in fermentable carbon sources like glucose and fructose. Accordingly, deletion of *TPS1* results in loss of trehalose accumulation. Synthesis of trehalose does not occur in Δ*tps1* mutants, and any intracellular trehalose detected in these cells is likely a result of its uptake from the YPD medium. Uptake is largely dependent on the expression of *AGT1*, which encodes an α-glucoside transporter (Plourde-Owobi, *et al*., 1999).

Trehalose has been described as a reserve carbohydrate with a crucial role in stress tolerance and protection. Depending on the environmental conditions, it can constitute up to 25% of the dry mass of *S. cerevisiae*. Accumulation of trehalose increases stress resistance of yeast growing on non-fermentable carbon sources (Van Dijck, *et al*., 1995), and resistance to freezing (Soto, *et al*., 1999) . In *S. cerevisiae*, endocytosis is inhibited in the presence of 2 to 6% (v/v) ethanol. This inhibition is accompanied by an accumulation of the stress protectors trehalose and Hsp104p, both important for ethanol stress resistance (Lucero, *et al*., 2000). Trehalose may also protect membranes from desiccation, maintaining membrane integrity by substituting water molecules and binding to the polar head-groups of phospholipids (Crowe, *et al*., 1992). Intracellular trehalose accumulates in response to menadione stress, but when cells are exposed to H_2O_2 it is only required outside the plasma membrane. When both stresses are present simultaneously, trehalose protection is required on both sides of the lipid bilayer, preventing lipid oxidation of both the outer and inner layers of the plasma membrane, as well as oxidation of proteins (da Costa Morato Nery, *et al*., 2008).

When studying resistance to freezing, Pacheco and co-workers (Pacheco, *et al*., 2009) observed that the intracellular concentration of trehalose was 50% higher in *hsp12*Δ cells than in the *wild-type* strain. Hsp12p is a small heat-shock protein described

as a Late Embryogenic Abundant LEA-like protein in *S. cerevisiae* (Mtwisha L., *et al*., 1998) and is induced in cells exposed to heat shock, osmotic, oxidative, and ethanol stresses. Nevertheless, *hsp12*Δ mutants were more resistant to freezing in prolonged storage at – 20º C than Wt cells. This was explained by the higher intracellular trehalose concentration observed in these mutants. Synthesis and accumulation of trehalose, along with glutathione, has also been reported as a protection mechanism used by cells under stress caused by high pressure (Yongsheng Dong 2007).

In summary, the accumulation of trehalose has been implicated in the tolerance to several stresses. However, if high levels persist during recovery from stress, it can lead to inactivation of important yeast enzymes such as glutathione reductase, that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, an important cellular antioxidant. (Sebollela, *et al*., 2004). A fine-tuned balance is therefore needed between trehalose synthesis and degradation.

1.3.2 The cell stress response to ethanol

Ethanol constitutes the main source of stress during fermentation. After exposure to sub-lethal concentrations of ethanol, yeast develops molecular mechanisms that increase tolerance to ethanol stress. Thus, the response of yeasts to environmental stress is complex, and various aspects of cell sensing, signal transduction, transcriptional and posttranscriptional control, protein-targeting, accumulation of protectants, and increased activity of repair functions are involved (Mager and Ferreira, 1993). Similarly to heat shock, ethanol promotes the expression of HSPs (Heat Shock Proteins), although their role in this type of stress is not well understood (Mahmud, *et al*., 2010).

Some studies have suggested that the lipid composition and fluidity of the plasma membrane is the basis for ethanol resistance (Alexandre, *et al*., 1994b; You, *et al*., 2003). In addition, five genes related to the integrity of the cell wall (*BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2*) are required for ethanol tolerance (Takahashi, *et al*., 2001). Mutants lacking mitochondrial manganese-superoxide dismutase (MnSOD) are also sensitive to ethanol, indicating that *SOD2* is essential for ethanol tolerance (Costa, *et al*., 1997).

The toxicity of alcohols is directly related to their lipophilicity. Lipophilicity is represented by log P_{ow} (logarithm of the octanol and water partition coefficient of a

solvent) and is used to estimate the toxicity of alcohols. Fujita and co-workers showed that this correlation is applicable to the yeast *Saccharomyces cerevisiae*, and also showed that lipophilic alcohols with high log P_{ow} values were more toxic to yeast (Fujita, *et al*., 2004). Based on this correlation, these authors decided to study the genes required for tolerance to ethanol and other alcohols. The screen identified 137, 122 and 48 mutants clearly more sensitive to ethanol, 1-propanol, and 1-pentanol, respectively, and 33 of the mutants were more sensitive to all three alcohols. All genes classified as having a vacuolar function (13) conferred co-sensitivity to ethanol,1-propanol and 1-pentanol. It is known that the vacuolar H^+ - ATPase (V-ATPase) pump acidifies intracellular vacuolar compartments (Stevens and Forgac, 1997). Interestingly, of the 13 deletion mutants, seven genes were involved in hydrogen-transporting ATPase activity and 10 genes were involved in vacuolar acidification. This suggests that V-ATPase function is fundamentally required for alcohol tolerance. Interestingly, more genes are needed for tolerance to alcohols with lower toxicity, such as ethanol. In fact, the number of genes required for tolerance to alcohols seems to be closely related to their lipophilicity (or toxicity). It was observed that the mutants sensitive to 1-propanol and 1-pentanol were also ethanol- sensitive. These results imply that a certain core set of genes is required for tolerance to various alcohols, regardless of their lipophilicity (or toxicity).

Alexandre and co-workers analyzed global gene expression during a short-term ethanol stress in *S. cerevisiae* and clustered the genes with altered expression into functional classes according to Gene Ontology (Fig.1) (Alexandre, *et al*., 2001). This distribution clarified the molecular mechanisms that promote ethanol tolerance. Down-regulated genes were mostly involved in cell growth, cellular biogenesis, protein biosynthesis and RNA metabolism. Down-regulation of these genes could be a reflex of the growth arrest that occurs in the presence of stress, which allows the cell to save energy and develop adaptation mechanisms. However, the genes that were up-regulated are mainly involved in energetic metabolism, ionic homeostasis, protein trafficking and stress response. Another study reported another set of genes that are up-regulated during ethanol stress, the ESR family (environmental stress response) (Gasch, *et al*., 2000). Other genes reported in this study were the *SSA1, SSA2, SSA3, SSA4, SSE1* genes. They encode HSP70 family members, supporting the prediction that one of the main effects of ethanol is protein unfolding.

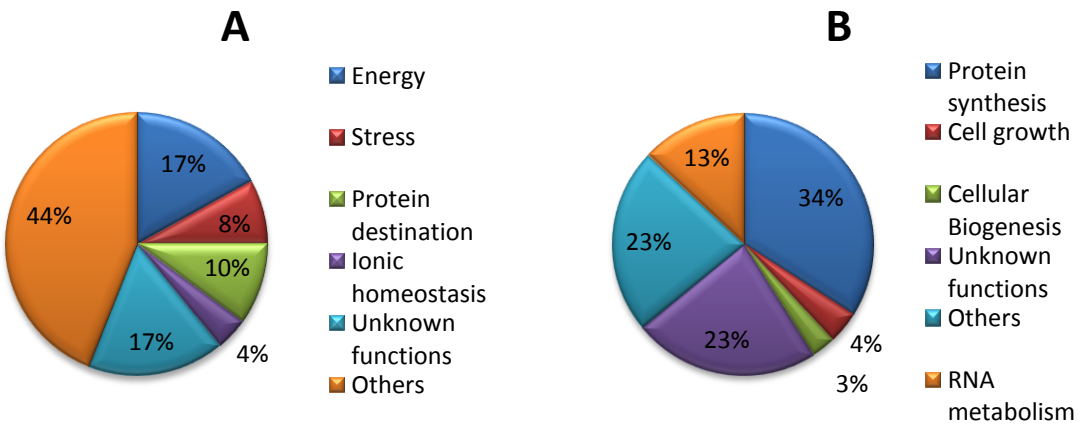

Fig. 1 - Distribution of ethanol-induced genes in the most representative classes. A: Induced genes, B: down-regulated genes (Adapted from Alexandre, *et al*., 2001)

Yet another study reported that genes encoding proteins involved in vacuolar function, cell integrity pathway, mitochondrial function, subunits of the co-chaperone complex GimC and components of the SAGA transcription factor complex were required for growth under ethanol stress (van Voorst, *et al*., 2006). A more directed study sough to determine whether the *MSN2*/*MSN4* pathway is activated in response to ethanol stress. The authors assessed the localization of a C-terminal Msn2-GFP fusion protein and found that, under ethanol stress, most of the fluorescence was accumulated in the nucleus, indicating there is activation of the general stress response. Similar results were observed with Ars1p (a yeast Ring／PHD finger protein that constitutively shuttles between the cytoplasm and nucleus), but curiously this translocation to the nucleus was observed under ethanol stress but not under heat, osmotic and oxidative stresses (Betz, *et al*., 2004).

TPS1 and other genes associated to trehalose synthesis including *TLS1*, *TPS2*, *UGP1* and *PGM2* were induced under ethanol stress (Alexandre, *et al*., 2001).These results were expected, as trehalose had previously been reported to accumulate in response to ethanol stress and its protective role against ethanol had been established (Mansure, *et al*., 1994) HSP genes were also induced under ethanol stress conditions (Alexandre, *et al*., 2001). Similarly to heat treatment, co-induction of trehalose and the HSP genes during ethanol stress supports the existence of a tight link between these two protective agents (Alexandre, *et al*., 2001; Winkler, *et al*., 1991). A model describing this interplay has been put forward (Singer and Lindquist, 1998) suggesting that in a

first step trehalose biosynthesis prevents proteins denaturation, subsequently HSP prevent protein aggregation, and finally the dissacharide is degraded because it impedes protein stabilization by HSPs.

Phosphorylation of the MAPK Slt2p has also been observed in response to ethanol (van Voorst, *et al*., 2006) This is in accordance with another study that concluded that ethanol is responsible for the activation of the PKC/Slt2 cell wall integrity pathway, and that deletion of genes encoding various components of this pathway result in ethanol sensitivity (Hirasawa, *et al*., 2007; Zu, *et al*., 2001). In addition, Takagi and co-workers reported that intracellular accumulation of proline might confer tolerance to ethanol stress in yeast, and defend that tryptophan can also contribute to ethanol tolerance (Takagi, *et al*., 2005).

1.3.2.1 – Does *Saccharomyces cerevisiae* have a specific ethanol stress response?

Although the involvement of general stress response mechanisms in ethanol stress has been described, some authors defend that there is an ethanol-specific response (Betz, *et al*., 2004; Takemura, *et al*., 2004). In *Saccharomyces cerevisiae,* ethanol stress causes selective mRNA export, similarly to heat shock. While analyzing the effect of stress on mRNA export factors, it was found that the DEAD box protein Rat8p showed a rapid and reversible change in its localization in cells treated with high concentrations of ethanol, accumulating in the nucleus. However, the localization of Rat8p did not change in heat-shocked cells, suggesting that changes in the localization of Rat8p contribute to the selective export of mRNA in ethanol stressed yeast cells, and indicating there are differences in mRNA export triggered by the heat shock response and the ethanol stress response (Takemura, *et al*., 2004).

Another ethanol-specific transcription regulator has been identified, Asr1. Asr1 is a yeast Ring / PHD finger protein that constitutively shuttles between the cytoplasm and nucleus. It accumulates in the nucleus rapidly and reversibly in response to alcohol stress (Betz, *et al*., 2004), but not to other stresses such as oxidative, osmotic, nutrient limitation, and heat. However, a specific role for Asr1p in response to ethanol stress was not supported by a different study, which confirmed this nuclear accumulation but did not find a phenotype associated with absence of Asr1p in ethanol tolerance (Izawa, *et al*., 2006). Further work is needed to determine if Asr1p is involved

in a specific alcohol stress response, as well as to identify a possible specific ethanol stress response in *S. cerevisiae*.

1.3.3 The cell stress response to weak carboxylic acids

Mollapour and Piper, (2006) studied the mechanisms involved in acetic acid resistance in *S. cerevisiae* by screening protein kinase deletion strains of *S. cerevisiae*. They found that the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase cascade is important for acetic acid resistance. In addition to the *hog1*Δ strain, there were three other acetic acid-sensitive deletion strains, *pbs2*Δ, *ssk1*Δ and *ctk2*Δ. From the kinases tested, only the loss of Hog1p generated acetate sensitivity, while the loss of the cell integrity MAP kinase Slt2/Mpk1 protein slightly increased acetate resistance. Based on these results, the authors concluded that the Hog1p mitogen-activated protein kinase determines acetic acid resistance in *S. cerevisiae*.

Recent studies have shown that when Hog1p is active it exerts important functions, for instance, at the plasma membrane, where it directly phosphorylates certain membrane ion transporters in osmostressed cells for rapid readjustment of the transmembrane fluxes of Na^+ and K^+ (Proft and Struhl, 2004). Mollapour and Piper (2006) also observed that Fps1p was a major factor in acetic acid resistance at low pH (4.5). Fps1 is an aquaglyceroporin, a membrane channel that mediates the flux of water and small solute molecules into and out of cells. Cells without Fps1p (*fps1*Δ) were more resistant to acetic acid on solid media than the wild type strain. Deleting *FPS1* rendered the activity of Hog1 MAPK, which is normally essential for resistance to acetic acid, dispensable. At low pH, accumulation of acetic acid was eliminated in acid-challenged cells without *FPS1*, which suggests that the entry of acetic acid in wild-type cells may occur by passive diffusion thought the Fps1p channel. In cultures at pH 6.8, the cellular accumulation of acetate was reduced in comparison to cultures at pH 4.5, which suggests that Fps1p facilitates the transmembrane flux of the undissociated form of acetic acid. In order to determine how Fps1p might be downregulated by Hog1p, the authors investigated if Fps1p levels were affected by acetic acid stress. Results showed that Hog1 MAPK activation by acetic acid directs endocytosis and degradation of Fps1p. It was concluded that Hog1p is responsible for the phosphorylation of Fps1p and

that this phosphorylation is the signal for the channel to be ubiquitinated and endocytosed to the vacuole.

Mollapour and Piper, (2006) also showed that both Hog1p and Slt2p MAP kinases are active in cell cultures exposed to 100 mM acetate. However, only active Hog1p is needed for acetate resistance. While pH affects the kinetics of Hog1p phosphorylation in response to acetic acid stress, a much smaller effect was observed on the commensurate phosphorylation of Slt2p. It is known that the Slt2 MAP kinase is involved in cell wall remodeling and in the maintenance of cellular integrity (Levin, 2005) and that the membrane sensors Wsc1–4p, Mid2p and Mtl1p are upstream components of this pathway. Of the different mutants lacking the membrane sensors, deletion of Wsc1p had the greatest effect on acetic acid resistance. The cell wall integrity signaling activated by acetate was strongly dependent on Wsc1p and enhanced the loss of negative regulation of Rho1p GTPase activity. In the presence of acetic acid stress, the Fps1p channel is essential for Hog1p activation but the activation of Slt2(MPK)p is suppressed.

A genome-wide identification of *S. cerevisiae* genes required for tolerance to acetic acid was performed by Mira and co-workers (Mira, *et al*., 2010). Several genes implicated in the homeostasis and uptake of glucose, iron, potassium and amino acids were identified, revealing the importance of the capacity of the cell to efficiently promote uptake and biosynthesis of these molecules. In addition, expression of genes involved in the biosynthesis of plasma membrane lipids, including phospholipids (*SUR4, CHO2, ARV1*), sphingolipids (*SUR1, SCS7*) and ergosterol (*ERG28, ERG4, ERG3, ERG2*), are essential to modulate the plasma membrane structure under acetic acid stress (Dickson, 2008; Hillenmeyer, *et al*., 2008; van der Rest, *et al*., 1995). Other genes required for tolerance to acetic acid are those encoding proteins involved in protein mannosylation (*MNN2* and *MNN9*) and in the activity and regulation of glucan synthase (*FKS1, ROM2, ROT2, BEM4*). Indeed, it has recently been proposed that glucan synthase is a biological target of acetic acid in yeast cells (Mollapour, *et al*., 2009).

28 transcription factors, including Haa1p, Rim101p, and Msn2p have also been described as required for the resistance to acetic acid (Mira, *et al*., 2010).

1.4. Aim

Previous works have shown there is an increase in cell viability when cells treated with high concentrations of ethanol are simultaneously exposed to sub-lethal concentrations of acetic acid, suggesting that low concentrations of acetic acid promote some type of response to ethanol stress (Vieira, 2001) (unpublished results from the lab).

Evidence of the involvement of the aquaglyceroporin Fps1p and of the MAP kinase Hog1p in acetic acid stress (Mollapour and Piper, 2006) triggered new studies to address the possible involvement of these proteins in the protector effect of acetic acid against ethanol stress. Several experiments on cell viability and analysis of membrane integrity were done, but deletion of these genes did not abrogate the protector effect. These observations suggested the protective effect of acetic acid against ethanol is independent of *HOG1* and *FPS1* (Trindade, 2009).

As referred above, both Hog1p and Slt2p are activated in the presence of acetic acid. Although deletion of *SLT2* renders cells more resistant to acetic acid this does not exclude the possible involvement of the cell wall integrity pathway in the protective effect of acetic acid from ethanol stress (Mollapour, *et al*., 2009).

The present work proposed to study which are the signaling pathways that are induced by sub-lethal concentration of acetic acid, other than HOG pathway, that play a role in the tolerance to ethanol, and to further characterize this protective effect. With this aim, cell viability and cytometric assays were performed on *Wt* cells exposed to 13% (v/v) ethanol and different concentrations of acetic acid. The influence of medium pH and the specificity of acetic acid in this process were also tested. The involvement of several stress response mechanisms in the acetic acid induced protective effect was addressed by the use of mutants, namely, *sfl1*Δ, *slt2*Δ, *tps1*Δ, *agt1*Δ, *hsp12*Δ and a respiratory deficient mutant (ρ_0), and by manipulating treatment conditions.

2. Materials and Methods

2.1. Yeast Strains

Strains used in this work were the Wt strain BY4741 and the mutants BY4741*slt2*Δ, BY4741 *sfl1*Δ, BY4741 *tps1*Δ, BY4741 *hsp12*Δ, BY4741 *agt1*Δ and BY4741 Rho^0 .

All strains have the same auxotrophic markers (*MATa his3*Δ*1 leu2*Δ0 met15Δ0 ura3Δ0-) and were obtained from EUROSCARF collection except BY4741 Rho^0 that was constructed (see 2.7.Transformation of BY4741 $Rho0^+$ strain in BY4741 Rho ^{0}).

2.2. Media and Growth Conditions

YPDA plates (1% yeast extract, 2% Bacto-peptone, 2% glucose and 2% agar) were prepared as previously described (Sambrook and Russell, 2001). Yeast cells were grown on YPDA plates at 30 °C for 2-3 days. After growth on YPDA, cells were then inoculated in Erlenmeyers with 10 ml of YPD medium, pH 3.5, at 26 ºC over-night (during 17-19 hours) with shaking (120 rpm).

2.3. Cell viability assays

For cell viability assays, 1.3 ml of $1*10^7$ cels/ml were collected in a microcentrifuge tube with a small hole in the lid. The cells were centrifuged and resuspended in 1.3 ml of YPD medium at pH 3.5.. Different cell viability assays were done using 13% (v/v) ethanol and several concentrations of acetic acid (0.0%, 0.025%, 0.05%, 0.1%, 0.2% and 0.4%). Ethanol and acetic acid used were obtained from stock solutions of absolute ethanol (> 99%, v/v) and of 1M acetic acid at pH 3.5. Cells were incubated at 26 ºC with shaking (120 rpm) for 3 hours. 50 µl samples were collected at different times (30, 60, 120, 180 min). After 3 dilutions (one dilution at 1:10 and two dilutions at 1:20), 7 drops of 40 µl of the last dilution were plated on YPDA. Cell viability was analyzed by counting colony forming units (c.f.u) after 2-3 days of growth

at 30 ºC. The percentage of survival for the different times was calculated by the formula: number of colonies in time X min (TX)/ number of colonies in time 0 (T0) x 100.

2.4. Flow cytometric assays

Integrity of the cell plasma membrane was analyzed by flow cytometry using the impermeable dye propidium iodide (PI) (Molecular Probes, SIGMA). Flow cytometry assays were performed with cells treated under the same conditions as described for cell viability assays. 50 µl samples were collected at specific intervals (120 and 180 min). Cells were resuspended in 475 µL PBS (1x) to which 2.5 µl of a PI stock solution (0.1 mg/mL) were added, followed by a 10 min incubation at room temperature in the dark. A cellular suspension treated with 70% (v/v) of ethanol was used as a positive control. Samples were analyzed in a Beckman-Coulter Epics XL cytometer and data analyzed with version 2.9 of the WinMDI software.

2.5. Assays with different acids at different concentrations

Different concentrations of acetic acid were tested to understand if the protective effect of acetic acid against ethanol stress was dependent on the concentration. To test the specificity of acetic acid, other weak monocarboxylic acids were tested in different concentrations. The acids and the concentrations used were:

- Formic acid (0.0125%; 0.025%; 0.05%; 0.1% v/v)
- Lactic acid (0.025%; 0.05%; 0.1%; 0.2% v/v)
- Propionic acid (0.025%; 0.05%; 0.1% v/v)

Viability and flow cytometry assays were performed as described above.

2.6. Assays at different pH values

To understand the influence of pH in the protective effect of acetic acid against ethanol stress, experiments in media at different pH values were performed. Experiments were done in YPD at pH 3.5, 5.0 and 6.0 with 13% ethanol (v/v) and 0.1%

(v/v) acetic acid from a stock solution of 1 M acetic acid at pH 3.5, 5.0 and 6.0, respectively. Viability and flow cytometry assays were performed as described above.

2.7. Transformation of the BY4741 Rho $^{+}$ strain in to BY4741 Rho ^{0}

$2x10^{8}$ cells/ml were grown in 10 ml YPD supplemented with 25 µl/ml ethidium bromide (SIGMA) for 10 days at 30 ºC with shaking. Cells were plated on YPDA in different dilutions (10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}), grown at 30 ºC for 2-3 days and replicated onto YPGA (1% yeast extract, 2% Bacto-peptone, 2% glycerol and 2% agar). Colonies that did not grow on YPGA plates were selected and the Rho^{0} phenotype tested by fluorescence microscopy using DAPI staining as described below.

2.8. Identification of a Rho ^{0} strain of *Saccharomyces cerevisiae*:

The presence of mitochondrial DNA was determined by staining with DAPI, 4',6-diamidino-2-phenylindole (Molecular Probes, SIGMA). A mid-log phase culture was harvested and washed with water and 50% (v/v) EtOH. DAPI was added at a final concentration of 0.1 mg/ml and cells were incubated at room temperature in the dark for 5 min and examined by fluorescence microscopy. Cells with blue fluorescence only in the nucleus have lost mitochondrial DNA and are considered Rho^{0}.

3. Results and Discussion

3.1. Acetic acid protects cells from ethanol-induced cell death in *Saccharomyces cerevisiae*

Ethanol is produced by *Saccharomyces cerevisiae* during alcoholic fermentation. Above a given concentration, it is responsible for a reduction of cell viability and inhibition of fermentation. During fermentation, some weak acids, such as acetic, butyric and pyruvic acids, present in the grape must or produced by yeast metabolism enhance ethanol toxicity and further inhibit growth and fermentation. However, previous data obtained in our lab showed that *S. cerevisiae* cells treated simultaneously with toxic concentrations of ethanol and low concentrations of acetic acid (0.1%, v/v) displayed higher survival (measured either by CFU or by propidium iodide staining) when compared to cells treated only with ethanol (Trindade, 2009). In this thesis, we aimed to understand how acetic acid protects cells from ethanol-induced death. We confirmed these results by assessing both cell viability by CFU counting and plasma membrane integrity of *S. cerevisiae* BY4741 cells exposed to 13% (v/v) ethanol and 0.1% (v/v) acetic acid, pH 3.5, for 3 hours (Fig.2). In Figure 2A we observe there was a higher percentage of cell viability in cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid than in cultures treated only with ethanol. Similar results were obtained by assessing plasma membrane integrity, although this loss was delayed in comparison to the loss of CFUs (Fig. 2B).

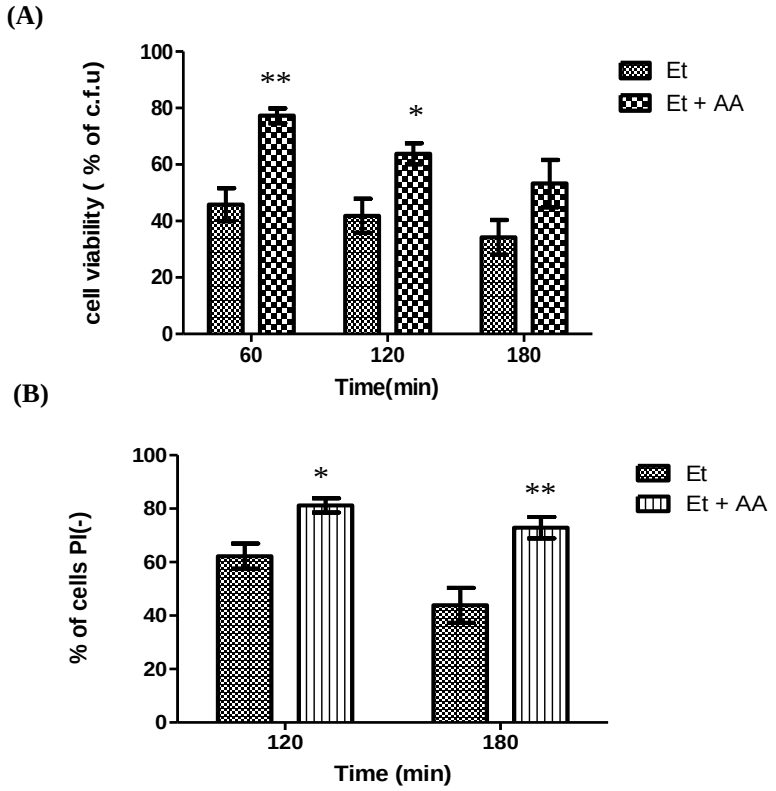

Fig.2 - Acetic acid protects BY4741 *Wt* cells from ethanol cytotoxicity. (A) - Graphic representation of cell viability. The values represent the average of 6 independent experiments and the respective standard deviations. The differences between different cultures are statistically significant for time 60 min ($P< 0.01$) and 120 min ($P<0.05$). (B) - Graphic representation of the percentage of cells that maintain plasma membrane integrity. The values represent the average of 13 independent experiments and the respective standard deviations. The differences between different cultures are statistically significant for time 120 min ($P< 0.05$) and 180 min ($P<0.01$). All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% ethanol (Et) or co-incubated with 13% ethanol and 0.1% acetic acid (Et + AA), for 3 hours.

3.1.1. The protective effect of acetic acid depends on its concentration

The next step was to ascertain whether this protective effect was concentration-dependent and to find which concentration of acetic acid resulted in the highest protection. Experiments with 13% (v/v) ethanol and different concentrations of acetic acid (0.025%, 0.05%, 0.1%, 0.2% and 0.4% (v/v), pH 3.5) were performed by assessing plasma membrane integrity (Fig. 3). Co-incubation of ethanol-treated cells with 0.05% or 0.2% (v/v) of acetic acid for 120 min and 180 min increased the percentage of cells with uncompromised plasma membrane integrity (propidium iodide negative stained cells, PI-). However, these results were not statistically significant. The lower acetic acid concentration tested (0.025%, v/v) had no significant protective effect against loss

of plasma membrane integrity induced by ethanol. After 180 min, the higher acid concentration (0.4%, v/v) enhanced the toxic effect of ethanol, thought after 120 min there was no effect. The results obtained with different concentration of acetic acid suggest the observed protective effect is dose dependent and highest at 0.1% (v/v), and above this concentration the acid enhances ethanol-induced death.

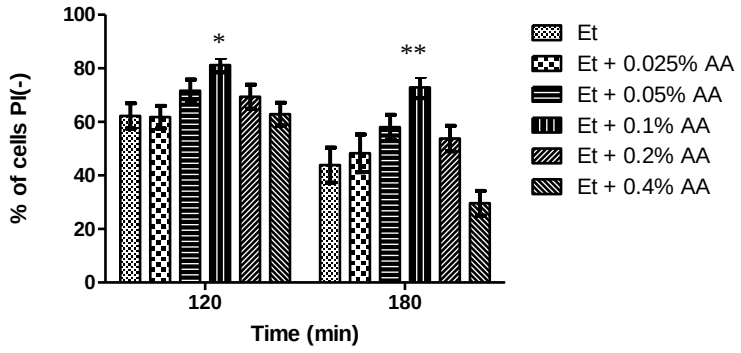

Fig.3 - The protective effect of acetic acid against ethanol stress. Graphic representation of the percentage of BY4741 *Wt* cells with preserved plasma membrane integrity after exposure to 13% (v/v) ethanol and different concentrations of acetic acid (0.025%, 0.05%, 0.1%, 0.2% and 0.4%) for 3 hours. Experiments were performed in YPD medium at pH 3.5. The values represent the average of 13 independent experiments and respective standard deviations. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant only for the concentration 0.1% acetic acid at time 120 min ($P< 0.05$) and 180 min ($P<0.01$). Legend: Et – Ethanol ; Et + AA – Ethanol and acetic acid.

3.1.2. The protective effect depends on the undissociated form of acetic acid

All experiments in the previous sections were performed in YPD medium adjusted to pH 3.5. At low pH values, and in glucose repressed cells the undissociated form of the acid predominates and may permeate the plasma membrane by simple diffusion (Casal, *et al*., 1996; Russell and Gould, 2003). Recently, it has been described that acetic acid may also enter yeast cells by a process of facilitated diffusion, mediated by the aquaglyceroporin Fps1p (Mollapour and Piper, 2007). Once inside the cell the acid may dissociate and disturb the internal pH homeostasis, which has consequences on lipid organization and in the function of cellular membranes. To understand the influence of pH in the protective effect of acetic acid against ethanol stress and its possible dependence on the undissociated form of acetic acid, several experiments were

performed by assessing cell viability and plasma membrane integrity at two higher pH values.

Cell viability of cultures exposed to 13% (v/v) ethanol and 0.1% (v/v) acetic acid in YPD medium, at pH 5.0 and pH 6.0, was higher than that of cultures treated only with ethanol. These differences were particularly evident after 180 min of treatment, although they are not statistically significant (Fig. 4A). Assessment of plasma membrane integrity under the same conditions showed that acetic acid did not protect cells from ethanol toxicity at pH 5.0 and 6.0 (Fig. 4B).

At pH 3.5 acetic acid is mostly in the undissociated form (94.68 %) whereas at pH 5.0 and 6.0 the dissociated form predominates (64.01% and 94.68 %, respectively). The absence of the protective effect at the higher pH values in comparison to pH 3.5 may be due to the much lower concentration of undissociated form of acetic acid. To discard a possible effect of the extracellular pH *per se,* the total acid concentration should be adjusted so that the cells are exposed to the same concentration of the undissociated form at the three pH values tested.

Overall, for the different incubation medium at both pH 5.0 and 6.0, the percentage of cells with loss of plasma membrane integrity was lower, than that obtained at pH 3.5 (Fig. 2B). As referred above, when the pH is low, proton release upon dissociation of the acid at the higher intracellular pH can promote acidification of the cytosol. If this is the case, metabolic functions (Krebs, *et al*., 1983) membrane lipid organization and function of cellular membranes are affected. This could explain the higher resistance observed in cells treated with ethanol and acetic acid at pH 5.0 and pH 6.0 than at pH 3.5.

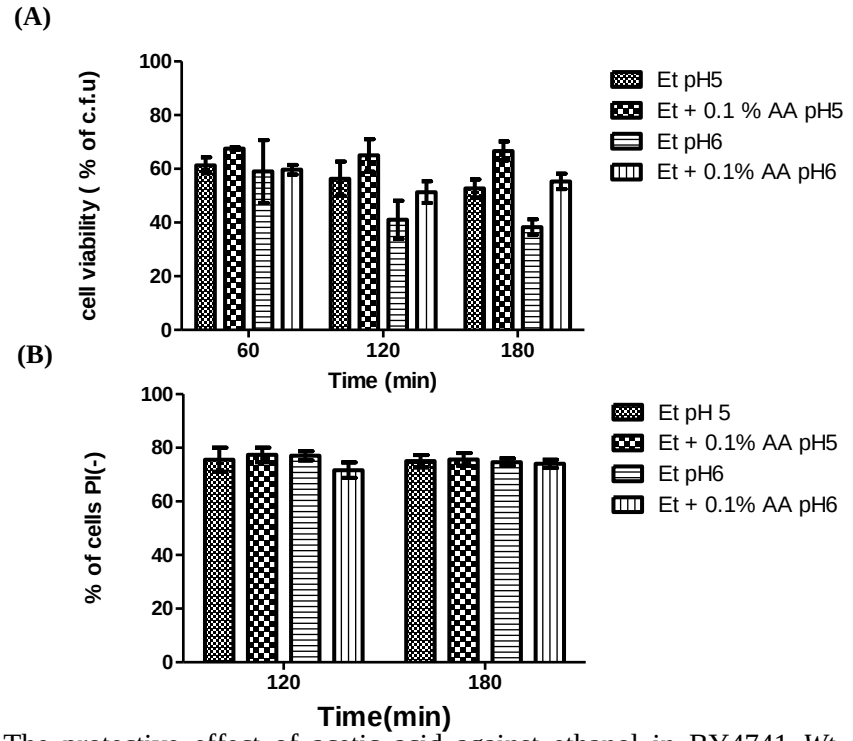

Fig. 4 - The protective effect of acetic acid against ethanol in BY4741 *Wt* cultures. (A) - Graphic representation of cell viability. The values represent the average of 3 independent experiments. (B) - Graphic representation of the percentage of cells that maintain plasma membrane integrity. The values represent the average of 3 independent experiments and the respective standard deviations. All experiments were performed in YPD medium at pH 5.0 and pH 6.0. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, for 3 hours. The acetic acid used was obtained from stock solutions of 1M acetic acid at pH 5.0 and pH 6.0 respectively Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

Since loss of plasma membrane integrity after exposure to 13% ethanol at pH 5.0 and pH 6.0 were low, the concentration of ethanol was increased in order to determine if for higher percentages of cell death there were differences between cells treated only with ethanol or with both ethanol and acetic acid at pH 5.0 and pH 6.0.

Cells were exposed to ethanol concentrations of 15%, 16%, 17%, 19% (results not shown) and 21% (v/v), alone or simultaneously with 0.1% (v/v) acetic acid at pH 5.0 and 6.0 (Fig. 5). Significant differences in loss of plasma membrane integrity between cultures treated with ethanol and ethanol and acetic acid were only visible when 21% (v/v) ethanol was used. As expected, loss of plasma membrane integrity was higher in cells exposed to 21% (v/v) ethanol than in cells exposed to 13% (v/v) ethanol. However, cultures treated with 21% (v/v) ethanol and 0.1% (v/v) acetic acid had an even higher loss of membrane integrity, in contrast with the results obtained with co-incubation of acetic acid at pH 3.5. These results are in agreement with the results

obtained with 13% (v/v) ethanol at pH 5.0 and 6.0, suggesting that the protective effect of acetic acid against ethanol stress observed at the plasma membrane level depends on the concentration of undissociated form of the acid and may be influenced by the pH.

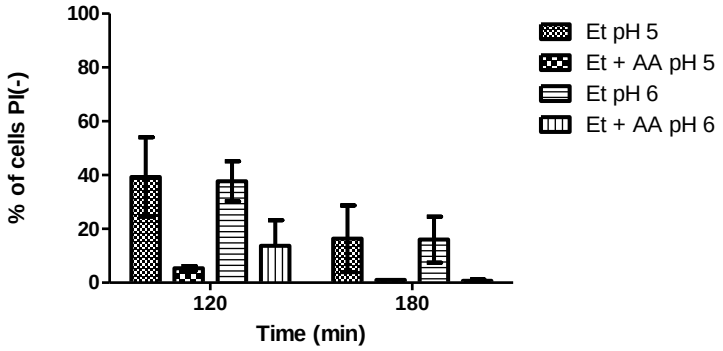

Fig. 5 - The protective effect of acetic acid against ethanol in BY4741 *Wt* cultures. Graphic representation of the percentage of cells that maintain plasma membrane integrity. The values represent the average of 3 independent experiments and respective standard deviations. All experiments were performed in YPD medium at pH 5.0 and 6.0. Cells were incubated with 21% (v/v) ethanol or co-incubated with 21% (v/v) ethanol and 0.1% (v/v) acetic acid, for 3 hours. The acetic acid used was obtained from stock solutions of 1M acetic acid at pH 5.0 and pH 6.0, respectively. Legend: Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.1.3. Specificity of the protective effect: the role of other weak carboxylic acids

Another question raised in this work was whether acetic acid specifically protects cells from ethanol stress or if other weak acids had the same effect. Therefore, we tested the effect of different concentrations of other weak carboxylic acids, namely formic, lactic and propionic acids.

Formic Acid

Formic acid is mainly used as a preservative and antibacterial agent in livestock feed and is a major ingredient of antiseptics. In *S. cerevisiae*, formic acid induces apoptosis and ROS production (Du, *et al*., 2008).

Wt BY4741 cells were exposed to 13% (v/v) ethanol and different concentrations of formic acid (0.0125%, 0.025%, 0.05% and 0.1%, v/v), pH 3.5(Fig. 6).

All concentrations of formic acid used in the experiments promoted a higher loss of plasma membrane integrity in comparison with cultures treated only with ethanol. The higher toxicity was observed even for very low concentrations of formic acid. This indicates that formic acid show this acid does not protect cells from ethanol stress.

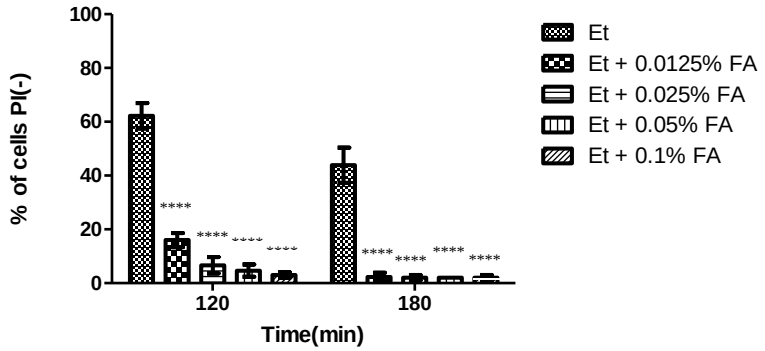

Fig. 6 - The protective effect of formic acid against ethanol stress. Graphic representation of the percentage of BY4741 *Wt* cells with preserved plasma membrane integrity after exposure to 13% (v/v) ethanol and different concentrations of formic acid (0.0125%, 0.025%, 0.05% and 0.1%) for 3 hours. Experiments were performed in YPD medium at pH 3.5. The values represent the average of 3 independent experiments and respective standard deviations. The differences between cells treated only with ethanol and treated with ethanol and formic acid are statistically significant for all concentrations of formic acid at time 120 min and 180 min ($P<0.0001$). Legend : Et – Ethanol ; Et + FA – Ethanol and formic acid

Lactic Acid

Lactic acid can be produced by microbial fermentation (bacteria's and yeasts), and has a large number of applications. It is currently considered the most useful chemical in food industry, acting as a preservative, flavouring and acidulant, and in the pharmaceutical, textile and chemical industries (C. Åkerberg, *et al*., 2000 ; Varadarajan and Miller, 1999).

The possible protective effect of lactic acid against ethanol stress was analyzed by exposing cells to 13% (v/v) ethanol and different concentrations of lactic acid (0.025%, 0.05%, 0.1% and 0.2%, v/v), pH 3.5 (Fig. 7). There was a higher percentage of cells with loss membrane integrity in cultures treated with both lactic acid and ethanol, in comparison with cultures treated only with ethanol. This indicates that lactic acid does not protect cells from ethanol stress.

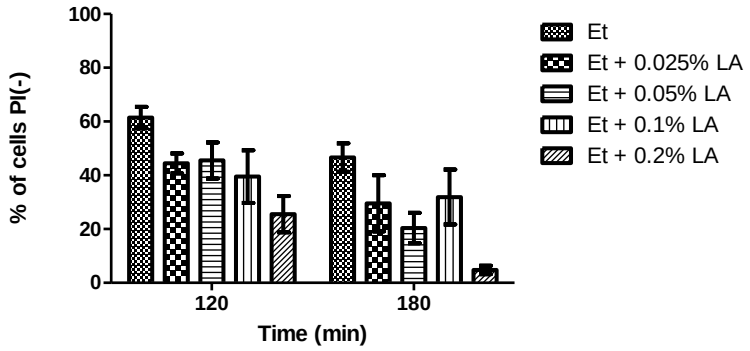

Fig. 7 - The protective effect of lactic acid against ethanol stress. Graphic representation of the percentage of BY4741 *Wt* cells with preserved plasma membrane integrity after exposure to 13% (v/v) ethanol and different concentrations of lactic acid (0.025%, 0.05%, 0.1% and 0.2%, v/v) for 3 hours. Experiments were performed in YPD medium at pH 3.5. The values represent the average of 12 independent experiments and respective standard deviations. Legend: Et – Ethanol; Et + LA – Ethanol and latic acid

Propionic Acid

Propionic acid is used as a preservative in bakery and fresh dairy products (Suhr and Nielsen, 2004). Several studies with the yeast *S. cerevisiae* have been performed in order to understand the mechanisms of tolerance developed in yeast cells exposed to propionic acid. It has been reported that cells exposed to propionic acid alter their cellular content of glutamate, trehalose and glycerol (Lourenço, *et al*., 2010).

Loss of plasma membrane integrity induced by 13% (v/v) ethanol and different concentrations of propionic acid (0.025%, 0.05% and 0.1%, v/v), pH 3,5, was assessed (Fig. 8). After 120 min, the percentage of cells with loss of membrane integrity was lower in cultures treated with both propionic acid and ethanol than that cultures treated only with ethanol, though the differences were not statistically significant. However, after 180 min, 80% of cells exposed to ethanol and 0.025% (v/v) propionic acid maintained plasma membrane integrity, while only 40% did so in cultures treated only with ethanol ($P<0.01$). 0.05% and 0.1% (v/v) propionic acid also reduced the percentage of cells with loss of plasma membrane integrity, in comparison with the cultures treated only ethanol, though to a lesser extent ($P< 0.05$).

The results showed propionic acid protects cells from ethanol stress. However, in contrast with acetic acid, the most significant differences were observed in cultures

treated with 0.025% (v/v) propionic acid. Based on these results, we confirmed that the protective effect against ethanol stress is not limited to acetic acid.

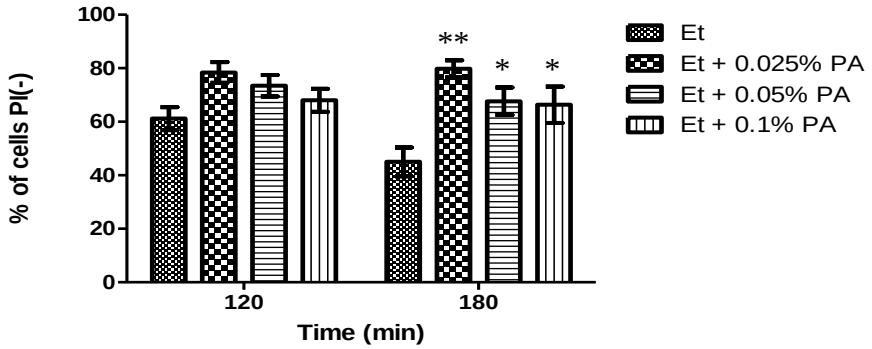

Fig. 8 - The protective effect of propionic acid against ethanol stress. Graphic representation of the percentage of BY4741 *Wt* cells with preserved plasma membrane integrity after exposure to 13% (v/v) ethanol and different concentrations of formic acid (0.025%, 0.05% and 0.1%, v/v) for 3 hours. Experiments were performed in YPD medium at pH 3.5. The values represent the average of 8 independent experiments and respective standard deviations. The differences between cells treated only with ethanol and treated with ethanol and propionic acid are statistically significant for all concentrations of lactic acid at time 180 min (for 0.025% $P< 0.01$, for 0.05% and 0.1% $P<0.05$). Legend: Et – Ethanol; Et + PA – Ethanol and propionic acid.

3.2. The protective effect of acetic acid against ethanol stress in the *slt2* mutant

When cells are exposed to some kind of stress, an appropriate response ensues. One frequently used molecular device for eliciting these responses is the three-tired cascade kinase (Mitogen-activated protein kinase - MAPK) module. MAPK pathways in yeast are involved in the pheromone response (Mating), filamentous growth, high osmolarity/glycerol pathway (HOG), cell wall integrity (PKC), and spore wall assembly. Five MAPKs mediate these responses (Gustin, *et al*., 1998). The four MAPKs present in vegetative cells, Kss1p, Fus3p, Hog1p and Slt2p/Mpkp1, are involved in the filamentation-invasion pathway, the mating-pheromone response, high osmolarity growth, and cell integrity pathway, respectively (Hahn and Thiele, 2002). The Slt2p/Mpk1p MAPK cell integrity pathway, is activated by several environmental stimuli such as hypoosmotic stress (Davenport, *et al*., 1995), mating pheromone (Zarzov, *et al*., 1996), heat shock (Martin, *et al*., 1993), actin depolymerization

(Harrison, *et al*., 2001), and agents causing cell wall stress (Ketela, *et al*., 1999). Activation of the MAP kinases, Hog1p and Slt2p has been observed in cell cultures exposed to acetate. However, only the activation of Hog1p is needed for acetate resistance (Mollapour and Piper, 2006). In the presence of acetic acid stress, the Fps1p channel is essential for Hog1p activity, however the activation of Slt2(MPK)p is suppressed (Mollapour, *et al*., 2009). The possible involvement of Hog1p and the Fps1p channel in the protective effect of acetic acid against ethanol stress had already been studied (Trindade, 2009). In this work, the possible involvement of Slt2p MAP kinase in this protective effect was analyzed.

Cell viability assays were performed with the BY4741 *slt2*Δ strain using 13% (v/v) ethanol and different concentrations of acetic acid (0.05% and 0.1%, v/v), pH 3.5 (Fig. 9) Deletion of an important MAP Kinase like Slt2p leads to a growth deficiency on YPD medium containing low concentrations of ethanol (van Voorst, *et al*., 2006). Therefore, a decrease in cell viability after exposure of the cells to 13% (v/v) ethanol was expected. However, this was not verified in our results. We observed that, the viability of BY4741*slt2*Δ cells exposed to ethanol was higher after 60 min of treatment than that of BY4741 Wt strain cells, though not significantly different for the other time points (compare Fig 2 and Fig 9). After 60 min of treatment the differences between cultures treated only with ethanol and cultures treated with ethanol and acetic acid simultaneously were not significant. After 120 and 180 min, the viability of cultures treated with ethanol and 0.1% (v/v) acetic acid was similar to that of cultures treated only with ethanol. However, there was an increase in viability of the culture treated with 13% (v/v) ethanol and 0.05% (v/v) acetic acid, when compared with cultures treated only with 13% (v/v) ethanol. The results show that the *slt2*Δ strain displays a protective effect similar to that obtained with the Wt strain. However, the acetic acid concentration that gives rise to the maximal protective effect is lower for the *slt2*Δ mutant than for the Wt strain (0.05%, v/v). Results suggest that the protective effect of acetic acid against ethanol-induced loss of cell viability is not abolished by deletion of *SLT2*.

Loss of plasma membrane integrity in cultures exposed to 13% (v/v) ethanol and different concentrations of acetic acid was tested, in order to determine whether acetic acid also protecs *slt2*Δ cells from ethanol-induced loss of plasma membrane integrity

(Fig. 9). However, the decrease in cell viability observed was not accompanied by loss of plasma membrane integrity. Indeed, the percentage of cells maintaining the integrity of plasma membrane during the 180 min of treatment was similar for all conditions tested and was very high (around 80%). Overall, BY4741 *slt2*Δ cultures exhibited a much higher percentage of cells maintaining plasma membrane integrity after 180 min of treatment than the Wt strain (compare Fig.9B and Fig.2B).

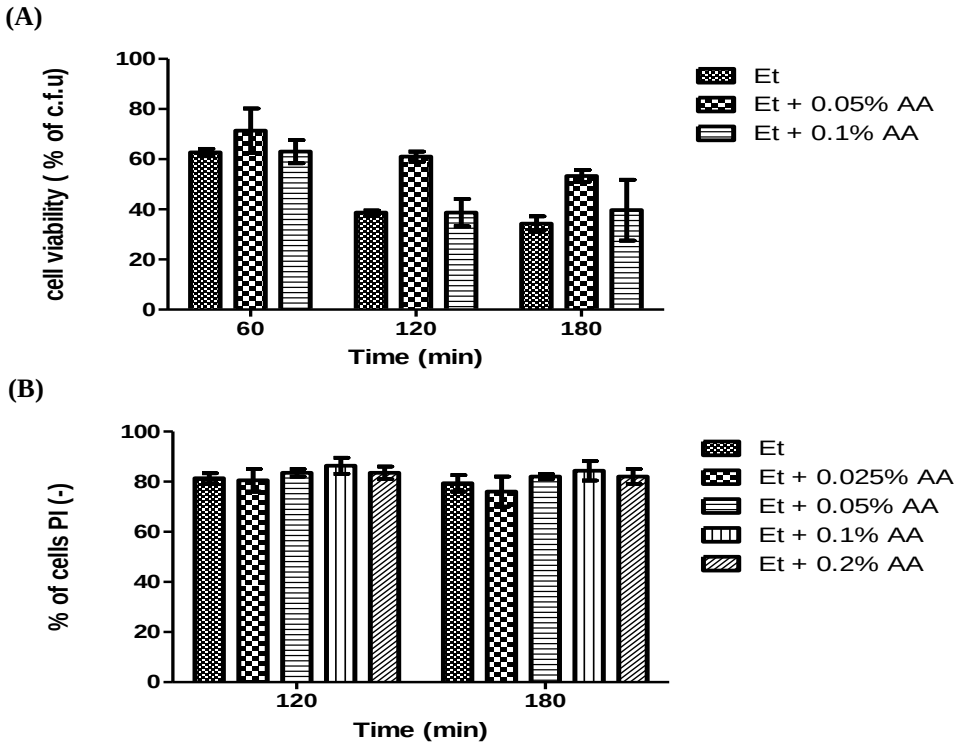

Fig. 9 - The protective effect of acetic acid against ethanol in BY4741 *slt2*Δ cultures. (A) - Graphic representation of cell viability. (B) - Graphic representation of cells that maintain plasma membrane integrity. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and acetic acid (0.05% and 0.1 %) (A) and (0.025%, 0.05%, 0.1% and 0.2 %)(B), respectively for 3 hours. All experiments were performed in YPD medium at pH 3.5. The values represent the average of 3 independent experiments and respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

Since no significant differences were obtained in the experiments with 13% (v/v) ethanol, additional assays were performed using 15% (v/v) ethanol and different concentrations of acetic acid (0.05% and 0.1%, v/v), pH 3,5 (Fig.10). As expected, the loss of membrane integrity was higher in cultures treated with 15% (v/v) ethanol than in cultures treated with 13% (v/v) ethanol. However, no significant differences were obtained between cultures treated with 15% (v/v) of ethanol and cultures treated with

15% (v/v) of ethanol and acetic acid, in accordance with the results obtained in the experiments performed with 13% ethanol.

These results suggest that Slt2p may be involved in the protective effect of acetic acid against ethanol –induced loss of plasma membrane integrity.

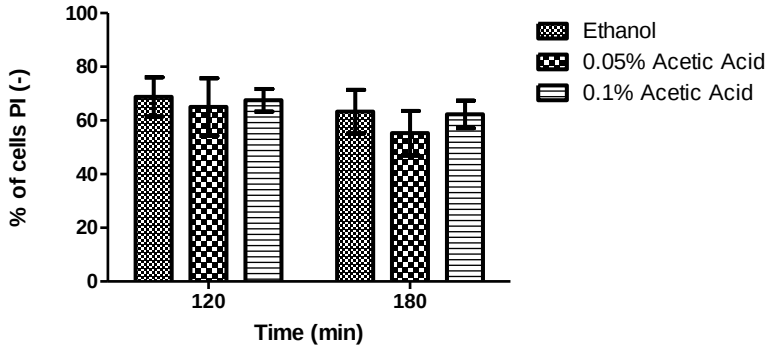

Fig. 10 - The protective effect of acetic acid against ethanol stress. Graphic representation of BY4741 *slt2Δ* cells with preserved plasma membrane integrity after exposure to 15% (v/v) ethanol and different concentrations of acetic acid (0.05% and 0.1%) for 3 hours. Experiments were performed in YPD medium at pH 3.5. The values represent the average of 4 independent experiments and respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.3. Protective effect of acetic acid against ethanol stress in the *sfl1* mutant

SFL1 was originally identified as a yeast suppressor gene for flocculation and it is required for normal cell-surface assembly in vegetative growth (Fujita, *et al*., 1989). It is negative regulated by cAMP-dependent protein kinase A subunit Tpk2p (Robertson and Fink, 1998) and recent studies showed that *SFL1* is involved in acetic acid stress resistance (Carvalho, 2009, results not published). We therefore decided to study whether Sflp is involved in the protective effect of acetic acid from ethanol stress. For this purpose, cell viability of *sfl1*Δ mutants was determined after exposure to 13% (v/v) ethanol and 0.1% (v/v) acetic acid for 3 hours (Fig.11A). After 60 mim, *sfl1*Δ mutant cells treated only with ethanol have a higher percentage of cell viability (74.3%) than wt cells (45.8%) under the same conditions (Fig. 2A). However, these differences disappeared after 120 min and 180 min. Co-incubation with acetic acid had no significant effect on cell survival. In parallel, loss of plasma membrane integrity was

evaluated in *sfl1*Δ mutant cells (Fig. 11B). Acetic acid did not significantly affect the percentage of PI (-) cells after 90 min treatment. Since *sfl1*Δ mutant cells were more resistant to ethanol, higher concentrations of this alcohol (14%, 15% and 18%., v/v) were tested. Since we had previously observed that the acetic acid concentration giving maximal protection could vary with the strain (Fig. 3 and Fig. 9B), different concentrations of acetic acid (0.025%, 0.05%, 0.1% and 0.2%, v/v) or 0.05% (v/v) for 15% and 18% (v/v) ethanol, respectively, were also tested.

Although no significant protective effect was obtained for any concentration of acetic acid, cultures treated with 0.05% (v/v) acetic acid displayed a lower percentage of cells with loss of plasma membrane integrity than cultures treated with the other concentrations of acetic acid (Fig. 11B). These results suggested this concentration of acetic acid could more adequate to detect a protective effect and was therefore tested with 18% (v/v) ethanol (Fig.12). *sfl1*Δ mutant cells treated with 18% ethanol and 0.05% acetic acid have 26% more PI(-) cells than cultures treated with only ethanol after 120 min ($P<0.001$) (Fig.12A). This difference was even more evident after 180 min, where cultures treated with ethanol and acetic acid have 38% more PI(-) cells than cultures treated only with ethanol ($P<0.0001$). These results suggest that Sfl1p is not involved in the protective effect of acetic acid against loss of plasma membrane induced by ethanol. Next, we tested whether 0.05% (v/v) acetic acid also protected *sfl1*Δ cells from loss of cell viability induced by 18% (v/v) ethanol.

*sfl1*Δ mutant cells displayed an increase of 20% in cell viability after 30 min of exposure to 18% (v/v) ethanol and 0.05% (v/v) acetic acid in comparison with cultures treated only with ethanol (Fig.12B). However, this difference was not detected after 60 and 120 min probably due to the low cell survival observed. Altogether, the results suggest that Sfl1p is also not involved in the protective effect of acetic acid against loss of cell survival by ethanol.

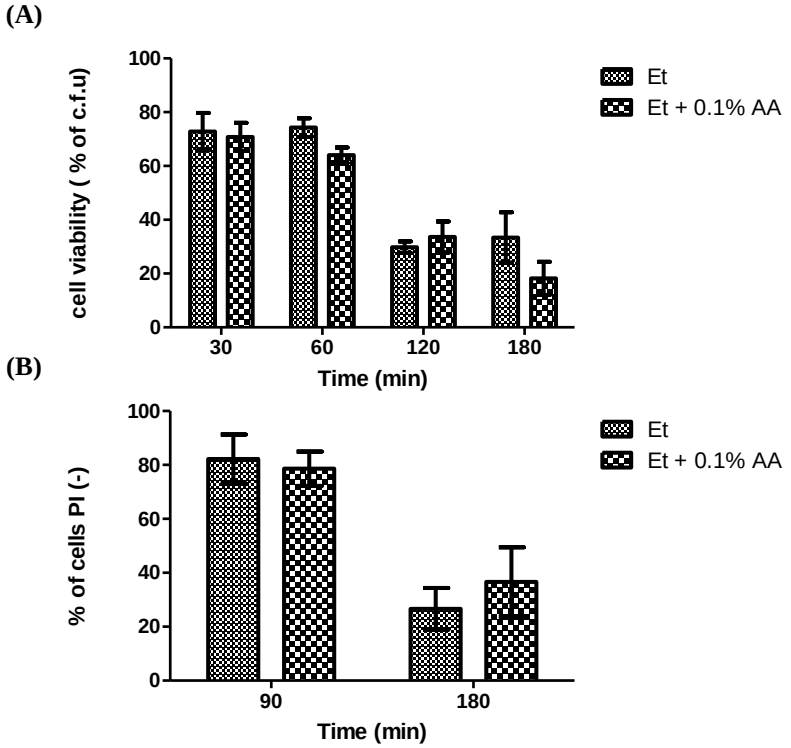

Fig. 11 - The protective effect of acetic acid against ethanol in BY4741 *sfl1Δ* cultures. (A) - Graphic representation of cell viability. The values represent the average of 5 independent experiments. (B) - Graphic representation of cells that maintain plasma membrane integrity. The values represent the average of 6 independent experiments . All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

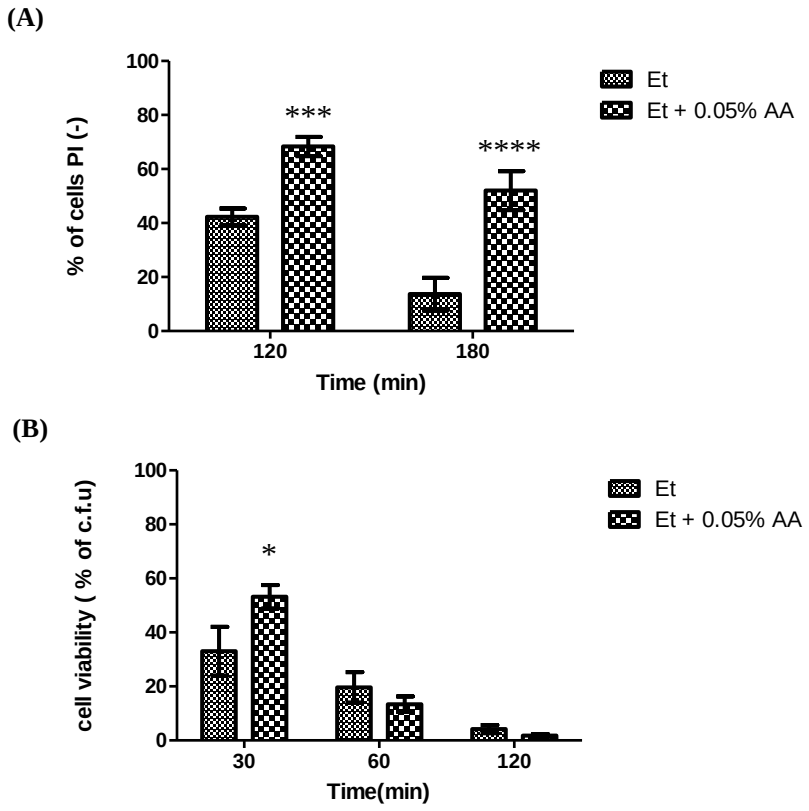

Fig. 12 - The protective effect of acetic acid against ethanol in BY4741 *sfl1Δ* cultures. (A) - Graphic representation of percentage of cells that maintain plasma membrane integrity. The values represent the average of 4 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 120 min ($P<0.001$) and 180 min ($P<0.0001$). (B) - Graphic representation of cell viability. The values represent the average of 3 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 30 min ($P<0.0001$). All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 18% ethanol and co-incubated with 18% ethanol and 0.05% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.4. The role of trehalose in protective effect of acetic acid against ethanol stress

The results presented in previous sections suggest that the protective effect of acetic acid against ethanol stress is only observed under very specific conditions, namely in YPD medium at pH 3.5. Moreover, preliminary assays suggested that it was not observed in SC medium. Among other constituents, YPD medium contains yeast

extract, which has high levels of trehalose. Analysis of a solution of yeast extract by proton nuclear magnetic resonance showed there are 113 mg of trehalose per g of yeast extract, resulting in a final trehalose concentration of about 0.3 mM in YPD medium containing 1% (w/v) of yeast extract (Xavier, *et al*., 1996). Trehalose, an important stress protector for yeast cells, is one of the most effective saccharides in stabilizing the membrane structure during desiccation (Crowe, *et al*., 1984) or exposure to high temperatures (Iwahashi, *et al*., 1995). Our preliminary results with SC medium raised the possibility that trehalose is involved in the protective effect of acetic acid against ethanol stress. Experiments using SC medium (1.7 g/L of Yeast Nitrogen Base without amino acids, 5 g/L ammonium sulfate and 2% (w/v) glucose) supplemented with BY4741 auxotrophic markers (100 mg/ L of methionine, histidine, uracil and 400 mg/l leucine) were performed at pH 3.5 during 3 hours with and without trehalose (0.3 mM). Cell viability and loss of plasma membrane integrity were analyzed and compared with the results obtained when YPD medium, pH 3.5, was used and are discussed below.

3.4.1. Experiments with the *Wt* strain

In contrast to YPD medium Wt BY4741 cultures in SC medium, treated only with 13% (v/v) ethanol had a higher percentage of cell viability than cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid, pH 3.5 (Fig.13A). It was also possible to observe that the viability of cultures treated only with 13% (v/v) ethanol in YPD and SC medium have a similar resistance to ethanol (compare Fig.2A and Fig.13A). However, the protective effect of acetic acid against ethanol stress was not verified when SC medium was used. In parallel loss of cells membrane integrity was evaluated in cultures treated under the same conditions (13% ethanol and 0.1% acetic acid in SC medium) (Fig.13B). In the cultures treated with 13% ethanol and 0.1% acetic acid after 120 min there was a decrease of 15% of PI(-) cells comparatively with cultures treated only with ethanol. This difference increased over time, being highest after180 min (24%) as for cell viability assays. As a whole the results confirmed that there is no protective effect of acetic acid against ethanol-induced death in SC medium.

We next tested if the addition of trehalose to SC medium could lead to a similar protective effect by acetic acid against ethanol as observed in YPD medium.

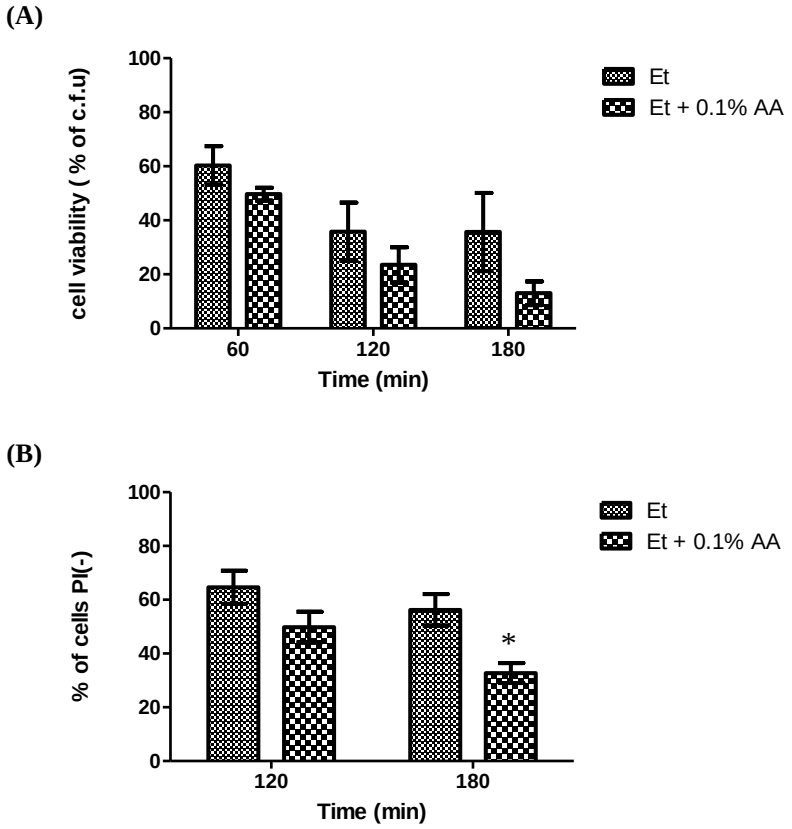

Fig. 13 - The protective effect of acetic acid against ethanol in BY4741 *Wt* cultures. (A) - Graphic representation of cell viability. The values represent the average of 4 independent experiments. (B) - Graphic representation of cells that maintain plasma membrane integrity. The values represent the average of 3 independent experiments and respective standard deviations. The differences between different cultures are statistically significant for time 180 min ($P< 0.05$) . All experiments were performed in SC medium with BY4741 auxotrophic markers at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

Indeed, adding trehalose to SC medium restored the protective effect by acetic acid against ethanol-induced cell. This effect increase along the time of the treatment and mimicked in the results obtained when YPD medium was used (Fig. 14). This represents the first evidence of the involvement of trehalose in the protective effect of acetic acid against ethanol stress. Suplementation SC medium with trehalose to resulted in an increase of 15 % of PI(-) cells in cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid in comparison to cells treated only with ethanol. This increase was identical to that observed in YPD under the same conditions (Fig. 2). Moreover, the loss of plasma membrane integrity appears to correlate with the loss of cell viability.

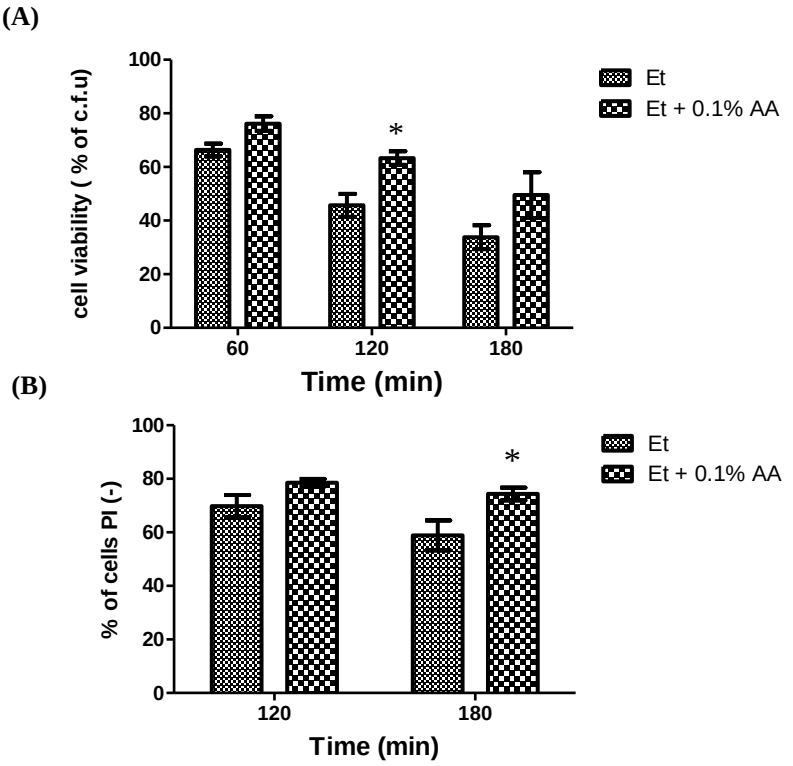

Fig. 14 - The protective effect of acetic acid against ethanol in BY4741 *Wt* cultures. (A) - Graphic representation of cell viability. The values represent the average of 4 independent experiments. The differences between different cultures are statistically significant for time 120 min ($P < 0.05$) (B) - Graphic representation of percentage of cells that maintain plasma membrane integrity. The values represent the average of 9 independent experiments. The differences between different cultures are statistically significant for time 180 min ($P < 0.05$). All experiments were performed in SC medium with BY4741 auxotrophic markers complemented with 0.3 mM trehalose at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.4.2. Experiments with the *tps1* mutant

Trehalose is biosynthesized in a two-step process, which involves trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase on a multimeric protein complex (see fig.22). The trehalose synthase complex is encoded by *TPS1* and *TPS2*, responsible for trehalose biosynthesis, and *TSL1* and *TPS3*, stabilizers of the trehalose synthase complex (Bell, *et al*., 1998; Vuorio, *et al*., 1993). Experiments performed with cells mutated in the genes responsible for trehalose biosynthesis have shown that the ability to synthesize trehalose and grow in glucose is lost only in cells without *TPS1*. All genes encoding subunits of the trehalose synthase complex present a significant degree of

homology to the *TPS1* gene. However, none of the other subunits replace the function of Tps1p in synthesizing Tre6P and in the control of glucose influx into glycolysis (Bell, *et al*., 1998).

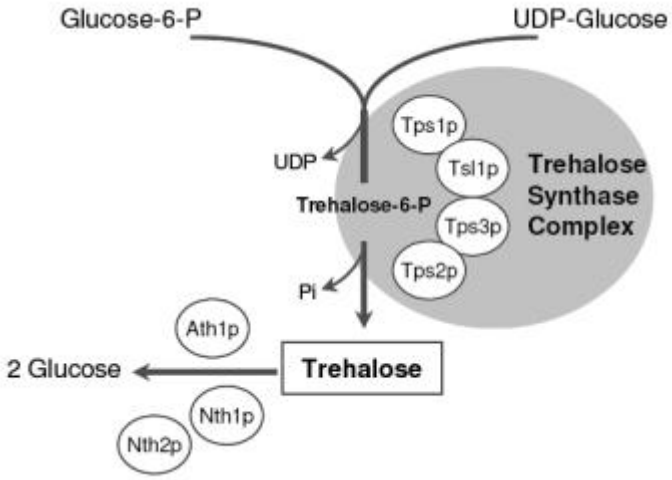

Fig. 15 - Trehalose metabolic pathways in the yeast *Saccharomyces cerevisiae* (Adapted from Jules, *et al*., 2004)

Since we showed that trehalose is involved in the protection of acetic acid against ethanol toxicity, cell viability and loss of plasma membrane integrity were evaluated in cultures of the trehalose synthase complex mutant BY4741 *tps1*Δ (Fig. 16). Initially, these assays were carried in YPD medium. The presence of acetic acid did not affect the loss of cell viability induced by ethanol after 180 min in *tps1*Δ cultures. These results indicate that absence of trehalose synthase activity reverts the protective effect of acetic acid against loss of cell viability. Although the percentage of PI(-) cells in *tps1*Δ mutant did not increase significantly when acetic acid was present, a small protective effect was reproduced for an n=4. This may be explained by the presence of trehalose in YPD medium. Therefore, experiments were repeated in SC medium supplemented with BY4741 auxotrophic markers, with and without trehalose.

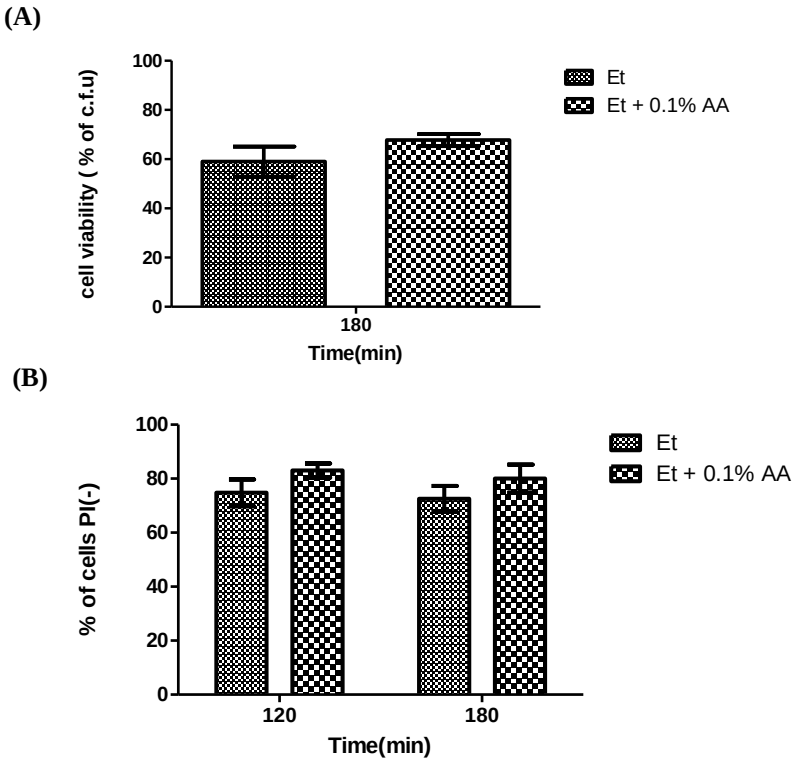

Fig. 16 - The protective effect of acetic acid against ethanol in BY4741 *tps1Δ* cultures. (A) - Graphic representation of cell viability. (B) - Graphic representation of cells that maintain plasma membrane integrity. The values represent the average of 4 independent experiments and respective standard deviations. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

The small increase in the loss of cell viability and plasma membrane integrity verified in *tps1*Δ cultures in YPD medium was not observed in SC medium under the same experimental conditions (Fig. 17A and 17B), suggesting that the residual protection was due to the YPD medium.

Next, to further confirm the importance of trehalose biosynthesis in this protective effect, experiments with SC medium supplemented with trehalose were carried out. If there is no reversion of phenotype in cells treated with ethanol and acetic acid in SC medium complemented with trehalose, then it may be concluded that trehalose biosynthesis is a crucial event that promotes the protective effect of acetic acid against ethanol stress. On the other hand, a reversion of phenotype would indicate the involvement of trehalose in this protective effect does not depend on its biosynthesis.

Results obtained with *tps1*Δ cultures in SC medium supplemented with trehalose showed an increased cell viability in cultures treated with ethanol and acetic acid in comparison with treatment only with ethanol (Fig.17C) . These results once again show that extracellular trehalose is important for the protective effect of acetic acid against ethanol stress and is not dependent on trehalose biosynthesis. Indeed cells without capacity to synthesize trehalose still show the protective effect of acetic acid against ethanol when trehalose is added to the culture medium. Moreover addition of trehalose to the medium seems to be even more important in conferring cell protection induced by acetic acid. However acetic acid did not protect *tps1*Δ mutant cells against loss of plasma membrane integrity under the same conditions indicating that trehalose synthesis seems to assume a protective role against loss of plasma membrane integrity induced by ethanol. However, the importance of trehalose transport was not assessed. In order to test the importance of trehalose transport in the protective effect of acetic acid against ethanol stress, cells defective in trehalose transport were used for the subsequent studies.

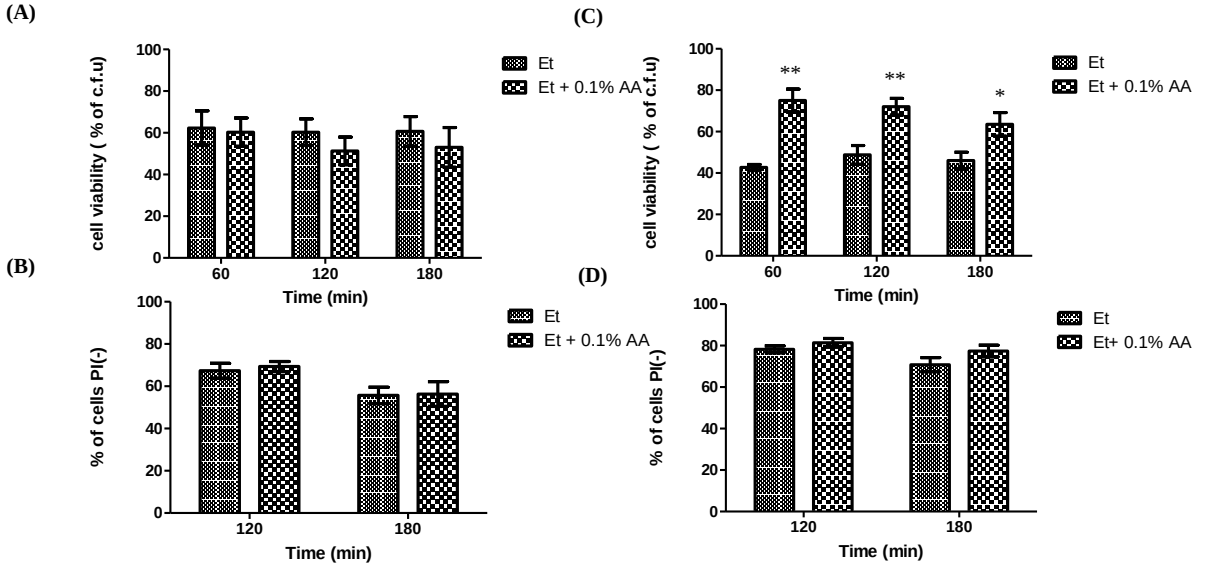

Fig. 17 - The protective effect of acetic acid against ethanol in BY4741 *tps1Δ* cultures. (A) - Graphic representation of cell viability. (B) - Graphic representation of cells that maintain plasma membrane integrity. Experiments were performed in SC medium with BY4741 auxotrophic markers at pH 3.5 (A and B).The values represent the average of 3 independent experiments. (C) - Graphic representation of cell viability. The differences between different cultures are statistically significant for time 60 min, 120 min ($P<0.01$) and 180 min ($P< 0.05$). (D) - Graphic representation of cells that maintain plasma membrane integrity. Experiments were performed in SC medium with BY4741 auxotrophic markers complemented with 0.3 mM trehalose at pH 3.5 (C and D).The values represent the average of 3 independent experiments. All cells cultures were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.4.4. Experiments with the *agt1* mutant

The mediated transport of trehalose was first described by (Kotyk and Michaljanicova, 1979) who characterized a high-affinity H^+–trehalose symporter, today known to be encoded by *AGT1*. Later (Stambuk, *et al*., 1996) described a facilitated diffusion mechanism Another pathway was also described associated with acid trehalase. It was first thought that trehalose reaches the vacuole by an endocytotic process, where it is degraded by the vacuolar acid trehalase (Nwaka, *et al*., 1996). Years later, it was reported that acid trehalase activity is extracellular, and cleaves the disaccharide into glucose in the periplasmic space (Jules, *et al*., 2004). So it is now proposed that secretion trehalase into the medium allows the hydrolysis of trehalose into glucose, which is then utilized by yeast cells for growth (Basu, *et al*., 2006).

To better understand the role of trehalose transport in the protective effect of acetic acid against ethanol stress, experiments with an *atg1*Δ mutant strain were performed. The Agt1 permease is a α-glucoside-H^+ symporter responsible for the active transport of trehalose and other sugars, such as sucrose, methylglucoside, maltose and maltotriose. Trehalose is the sugar preferentially transported by Agt1p, followed by sucrose (Stambuk, *et al*., 1999).

Cell viability and loss of plasma membrane integrity under our experimental conditions were evaluated in BY4741 *atg1*Δ cells (Fig.18). *agt1*Δ cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid displayed a higher percentage (74%) of viable cells than when treated only with 13% (v/v) ethanol (51%). These results suggest that the transport of trehalose mediated by Atg1p does not influence the protective effect of acetic acid against ethanol stress, *agt1*Δ cultures treated with 13% (v/v) ethanol and 0.1% acetic acid (v/v) had a similar percentage of cells with loss of plasma membrane integrity than cultures treated only with ethanol, which suggests that transport of trehalose by Agt1p is needed for the protective effect of acetic acid against loss of plasma membrane integrity induced by ethanol. The results for cell viability and plasma membrane integrity performed with *atg1*Δ cultures are similar to those obtained with *tps1*Δ cultures. In both cases, acetic acid did not protect against loss of plasma membrane integrity. Synthesis and transport of trehalose therefore seem to be important specifically for the protective effect of acetic acid against loss of plasma membrane integrity induced by ethanol but not for loss of cell viability. These studies did not

provide clues to understand how acetic acid can promote an increase cell viability of cells exposed to ethanol.

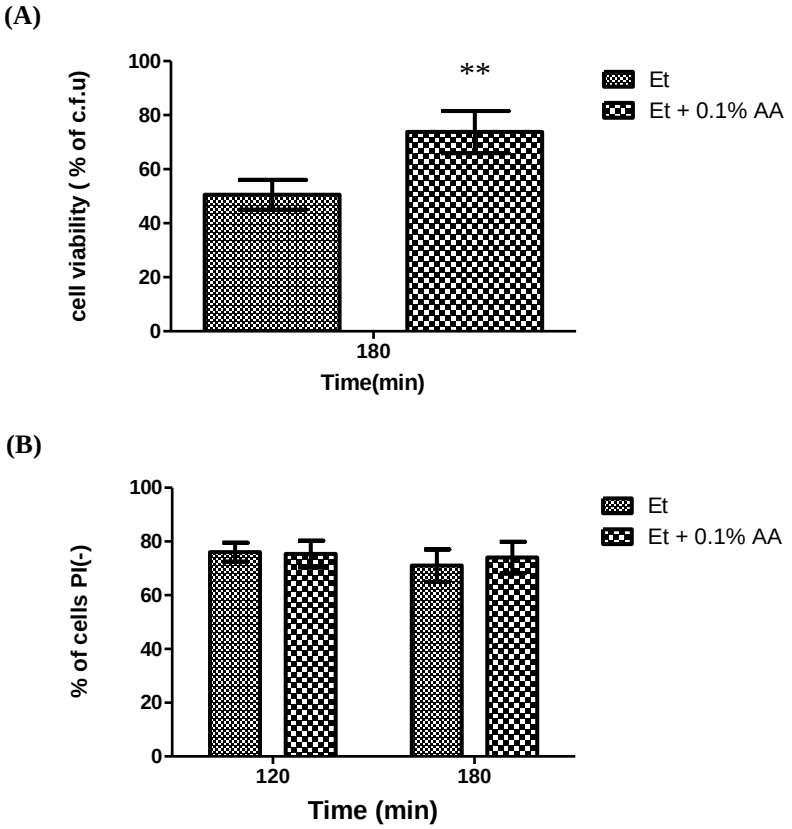

Fig. 18 - The protective effect of acetic acid against ethanol in BY4741 *agt1Δ* cultures. (A) - Graphic representation of cell viability. The values represent the average of 4 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 180 min ($P<0.001$) (B) - Graphic representation of the percentage of cells that maintain plasma membrane integrity. The values represent the average of 3 independent experiments. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.4.5. Experiments with the *hsp12* mutant

Hsp12p is a small heat-shock protein described as a Late Embryogenic Abundant (LEA)-like protein in *S. cerevisiae* (Mtwisha L., *et al*., 1998). It is induced in cells exposed to heat shock, osmostic, oxidative and ethanol stresses. It has been shown that *hsp12*Δ cells are more resistant to prolonged storage at – 20º C than the Wt strain. This was explained by the higher intracellular accumulation of trehalose in cells without

HSP12, as *hsp12*Δ cells had a 50% higher intracellular trehalose concentration than the *wild-type* strain (Pacheco, *et al*., 2009). In this work the authors concluded that trehalose and Hsp12p seem to have interchangeable role in cell protection during freezing storage. In light of these results we questioned if Hsp12p could be involved in the protective effect of acetic acid against ethanol stress, and therefore *hsp12*Δ cells were used for the subsequent studies.

The percentage of cell viability in *hsp12*Δ cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid was 12% higher than that of cultures treated only with ethanol, after 180 min (Fig. 19A). Although this difference is not very high, it was statistically significant. Compared with results obtained with the Wt strain, *hsp12*Δ cultures were more resistant to ethanol stress. The percentage of cell viability of Wt cultures treated only with 13% (v/v) ethanol for 180 was 34% whereas that of *hsp12*Δ cultures was 61%. These results were similar to those obtained when resistance to freezing was assessed (Pacheco, *et al*., 2009). It is therefore possible that trehalose accumulation in these mutants can render the cells more resistant to ethanol. The protective effect of acetic acid against ethanol-induced cell death does not seem to strictly depend on *HSP12*, however, the protective effect was less accentuated in *hsp12*Δ than in Wt cultures. *hsp12*Δ cultures displayed a lower percentage of cells with loss plasma membrane integrity after 3 hours of treatment that wild type strain (Fig. 19B). Once again these results may be explained by the high intracellular concentration of *hsp12*Δ strain.

Acetic acid did not protect *hsp12*Δ cells against ethanol-induced loss of plasma membrane. Altogether the results show that synthesis, transport and accumulation of trehalose are important events in such protective effect.

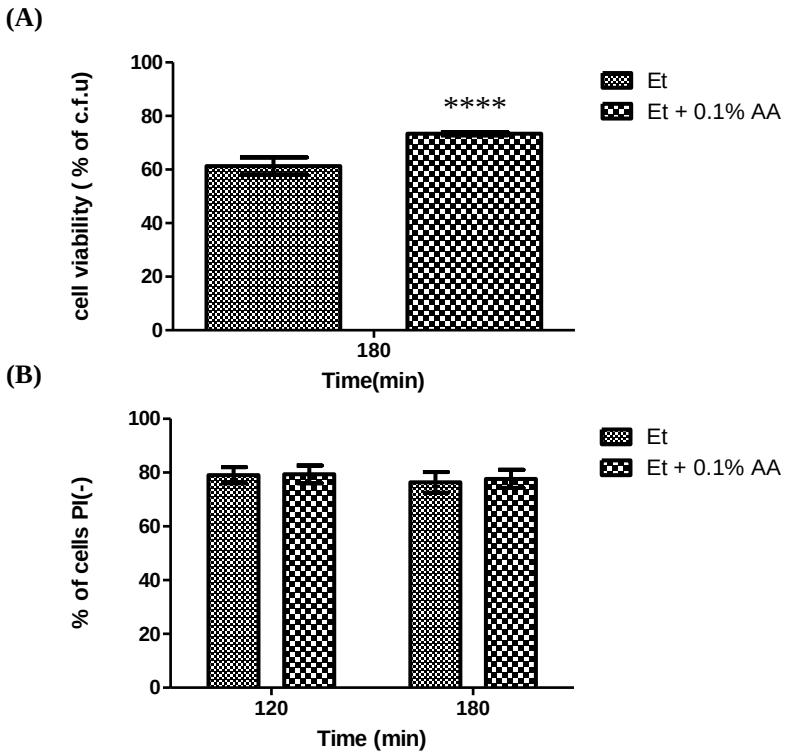

Fig. 19 - The protective effect of acetic acid against ethanol in BY4741 *hsp12Δ* cultures. (A) - Graphic representation of cell viability. The values represent the average of 4 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 180 min ($P<0.0001$) (B) - Graphic representation of percentage of cells that maintain plasma membrane integrity. The values represent the average of 3 independent experiments. All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% (v/v) ethanol and co-incubated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend: Et – Ethanol ; Et + AA – Ethanol and acetic acid

3.5. Protective effect of acetic acid against ethanol stress: the role of mitochondria

3.5.1 Experiments with Rho 0 mutant

Mitochondria are organelles found in all eukaryotic organisms, including yeast cells. Due to the ease of manipulation and growth, yeasts are used as a model system to study the biogenesis, function and structure of mitochondrial (Glick and Pon, 1995).The shape, size and number of yeast mitochondria vary with growth conditions, cell cycle phase, and strain backround, and are directly influenced by glucose concentration,

partial oxygen pressure, availability of fatty acids and sterols, and presence of unfermentable substrates, etc (Table 3).

Table 3 – Effects of nutrition on yeast mitochondria (adapted from Walker,1998).

Nutrient	Concentration	Oxygen	Respiration	Morphology
Glucose	excess	+	repressed	Few large
Ethanol	excess	+	activated	Many small
Glucose	excess	-	repressed	Few large
Glucose	limited	-	repressed	Few large
Glucose	limited	+	activated	Many small

.

Under aerobic conditions, yeast mitochondria are involved in ATP synthesis coupled to oxidative phosphorylation. The activities of the citric acid cycle and the respiratory chain largely depend on the yeast species and on the expression of the Crabtree effect. Under anaerobic conditions, mitochondria seem to be dispensable for respiratory functions. Petite mutants that lack functional mitochondrial respiration, also called ρ°, are viable. However, mitochondria have other components and physiological functions that are relevant to maintain cell metabolism, namely, mobilization of glycogen, enzymes for the synthesis of particular amino acids and dicarboxylic acids, pyrimididne and purine bases, porphyrin, and pteridines, synthesis and desaturation of fatty acids and lipids, biosynthesis of ergosterol, production of flavor components, and responses and adaptation to stress.

To evaluate the possible role of mitochondria in the protective effect of acetic acid against ethanol stress, ρ^0 mutants of BY4741 strain were first generated. For such cells were grown in the presence of ethidium bromide, to create so called BY4741 ρ^- cells. The absence of mitochondrial DNA in these cells was verified by DAPI staining to confirm the existence of ρ^0 cells (see Material and Methods). ρ^0 cultures treated with 13% (v/v) ethanol and 0.1% (v/v) acetic acid had a lower percentage of cell viability than cultures treated only with ethanol (Fig. 20A). After 180 min of treatment, the culture subject only to ethanol had 12% more viable cells than the culture treated with ethanol and acetic acid simultaneously. These differences are statistically significant, suggesting a possible involvement of mitochondria in the protective effect of acetic acid against ethanol stress. In comparison with the BY4741 Wt strain, ρ^0 cells were also much more resistant to ethanol stress alone, as 34% of wild type cells and 73% of ρ^0 cells were viable after exposure to 13% ethanol.

There was a lower percentage of cells that had lost plasma membrane integrity in BY4741 ρ^0 cultures treated with ethanol and acetic acid than in cultures treated only with ethanol (Fig. 20B). Although the difference between the cultures treated with and without acetic acid was only 5%, this difference is statistically significant, indicating the protective effect of acetic acid against ethanol stress still occurs in ρ^0 cells.

Similarly to the results obtained with cell viability assays, ρ^0 cells were more resistant to ethanol stress than the Wt strain when loss of plasma membrane integrity was evaluated. Whereas Wt cultures maintained the plasma membrane integrity in 44% of cells, 78% of ρ^0 cells cultures did not lose plasma membrane integrity after exposure to 13% (v/v) of ethanol. Contrary to the results obtained in the cell viability assays, the protective effect of acetic acid against ethanol stress was maintained at the plasma membrane level. These results suggest that mitochondrial function has a crucial role in the protective effect of acetic acid against ethanol stress, as cell viability is lower in cells treated with high concentrations of ethanol and low concentrations of acetic acid than in cell treated only with ethanol. This role does not seems to be important at the plasma membrane level, where the protective effect is still present in cells lacking functional mitochondria.

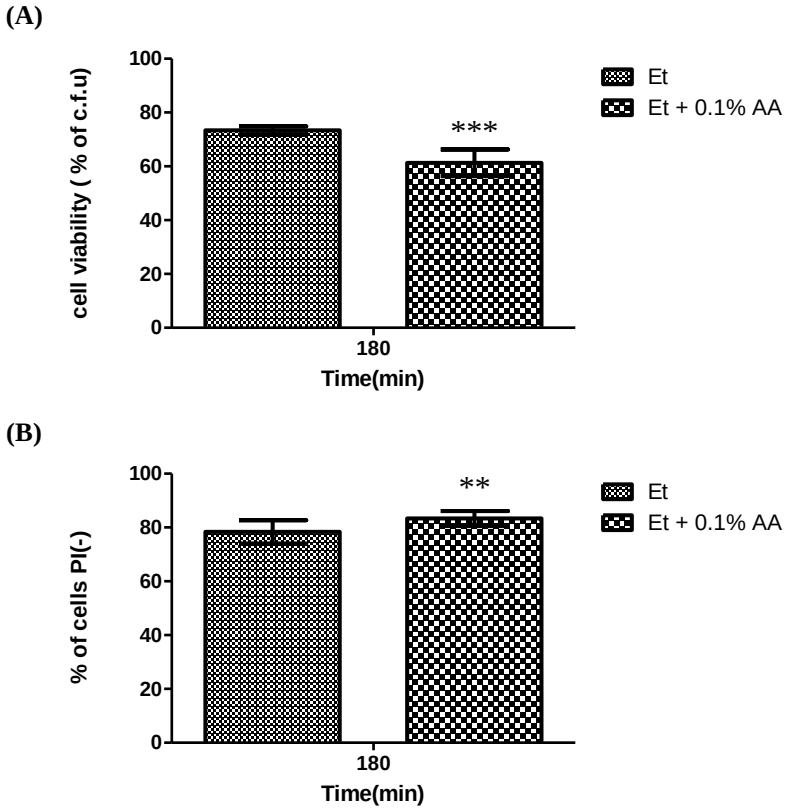

Fig. 20 - The protective effect of acetic acid against ethanol in BY4741 ρ^0 cultures. (A) - Graphic representation of cell viability. The values represent the average of 3 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 180 min ($P<0.001$) (B) - Graphic representation of percentage of cells that maintain plasma membrane integrity. The values represent the average of 6 independent experiments. The differences between cells treated only with ethanol and treated with ethanol and acetic acid are statistically significant at time 180 min ($P<0.01$). All experiments were performed in YPD medium at pH 3.5. Cells were incubated with 13% ethanol and co-incubated with 13% ethanol and 0.1% acetic acid, respectively for 3 hours. The values represent the respective standard deviations. Legend : Et – Ethanol ; Et + AA – Ethanol and acetic acid.

4. Conclusions and future perspectives

Ethanol, the main product of fermentative metabolism of yeast, may affect positively or negatively yeast performance depending on the culture conditions. In the absence of other carbon and energy source and at low concentrations it can promote growth under aerobic conditions. In the presence of glucose and sub-lethal ethanol concentrations it inhibits cell growth and division, whereas at high ethanol concentrations it induces cell death (Birch and Walker, 2000; Stanley, *et al*., 2010). Yeast have developed several stress response pathways that allow cells to cope with stress conditions induced by sub-lethal concentrations of ethanol such as changes in lipid membrane composition, metabolic activity and gene expression (Alexandre, *et al*., 1994b; Takahashi, *et al*., 2001; You, *et al*., 2003). Ethanol cytotoxicity has been attributed to different structural and functional cellular changes including lipids, membrane proteins and mitochondrial DNA, associated with oxidative stress, changes in vacuole and mitochondrial morphology and mitochondrial dysfunction, as well as inhibition of nutrient transport and metabolism. Yeast alcoholic fermentation by-products, such as octanoic and decanoic acids potentiate the harmful effects of ethanol and enhance ethanol induced cell death in *Saccharomyces bayanus* (Sa-Correia, 1986). Unexpectedly, previous work in our Lab has shown that sub-lethal concentrations of acetic acid protect against ethanol-induced cell death. Activation by acetic acid of a cell stress response against ethanol induced cell death or another cell alteration induced by acetic acid may counteract ethanol cytotoxicity, and explain such protection. Given the known deleterious effects of ethanol on yeast fermentative performance the understanding of protection by sub-lethal concentrations of acetic acid has a considerable impact in improvement of industrial processes such as bioethanol and wine production. Therefore this work aimed to ascertain the role of several signaling pathways induced by acetic acid in the protection of *S. cerevisiae* against ethanol.

This study shows that although different concentrations of acetic acid (0.05 and 0.2 % v/v acetic acid protect against ethanol-induced loss of plasma membrane integrity in a reproducible way, 0.1% (v/v) acetic acid is the concentration with the highest and statistically significant protective effect. Higher cell survival was also achieved in cultures exposed to ethanol and 0.1% (v/v) acetic acid than in cultures treated only with

ethanol. Acetic acid effects have been mostly attributed to the undissociated form of the acid which predominates at pH values lower than the pKa value. Accordingly, lower concentration of undissociated form of the acid at pH 5.0 and 6.0 do not protect against ethanol-induced loss of cell viability and plasma membrane integrity. In the future, it would be interesting to assess the effect at higher pH values keeping the same concentration of undissociated form in order to evaluate the effect of the extracellular pH alone.

The aquaglyceroporin Fps1p, can be involved in acetic acid entrance into the cell (Mollapour and Piper, 2007). However, it was shown that the protective effect does not dependent on this porin (Trindade, 2009). Whether protection requires acid up-take through passive diffusion and intracellular accumulation also deserves to be clarified. To this purpose acetic acid transport determinations by measurement of the uptake and accumulation of radioactive acetic acid in YPD medium at different pH (3.5, 5.0 and 6.0) should be performed.

Cometabolism of different mixtures of glucose and acetate or propionate using aerobic C-limited chemostats was reported (dos Santos, *et al*., 2003; Pronk, *et al*., 1994). Cytosolic acetyl-CoA synthetase and propionyl-CoA synthetase convert acetic and propionic acid to acetyl-CoA and propionyl-CoA, respectively, which may be used as precursors of fatty acids and amino acids. We therefore hypothesize that acetic and propionic acid protection may be related to the availability of biosynthetic precursors under ethanol stress conditions. Moreover, our observation that acetic acid under certain conditions protected against loss of plasma membrane integrity, but not against loss of cell viability was in line with our interpretation. To confirm this hypothesis and test whether the protection was specific of acetic acid, other carboxylic acids were tested. Though formic and lactic acids did not protect cells from ethanol stress propionic acid still display a protective effect against ethanol. These results indicate that the protective effect against ethanol stress is not specific to acetic acid. It would be interesting to test other weak carboxylic acids, such as intermediates of Krebs cycle, in other to identify other acids that promote this protective effect.

As afore mentioned Fps1p channel is not involved in protective effect of acetic acid, and on the contrary its deficiency lead to an increased protection effect by acetic acid. Moreover, deficiency of Fps1p leads to an hyperactivation of Slt2 (MPK)p. Therefore the role of this kinase was assessed in the present study. Viability of *slt2*Δ cultures treated with ethanol and 0.1% (v/v) acetic acid was not higher compared to

cultures treated only with ethanol. Nevertheless, when the concentration of acetic acid was reduced to 0.05% (v/v), the protective effect of acetic acid against ethanol-induced cell death was still evident. However, acetic acid did not protect ethanol-induced loss of plasma membrane integrity in this mutant. This suggests that Slt2/MPK1 may be involved in the protective effect of acetic acid by contributing to preserve plasma membrane integrity in response to damage caused by ethanol toxicity.

Accumulation of the disaccharide trehalose has been implicated in the tolerance to several stresses. Therefore the role of trehalose in the protection by acetic acid was analysed. Experiments performed with Wt cultures in minimal medium supplemented with trehalose showed similar results to those performed in YPD medium. However, when the experiments with Wt cultures were performed in minimal medium without trehalose, acetic acid did not protect but rather enhanced cell death and loss of plasma membrane integrity induced by ethanol indicating trehalose involvement in the protective effect. Strains affected in trehalose biosynthesis were also tested in different media. In YPD medium, *tps1*Δ cultures maintained the protective effect of acetic acid against ethanol-induced cell death and loss of plasma membrane integrity. However, this protection was minimal and the differences between cultures treated with and without acid were not significant. The presence of trehalose in YPD medium can provide an explanation for these results. In SC medium, *tps1*Δ cultures did not exhibit the protective effect of acetic acid, suggesting that trehalose biosynthesis is necessary to protect cells against ethanol toxicity. However, in SC medium, the same cultures suplemented with trehalose showed high levels of cell viability when treated with ethanol and acetic acid. We can conclude that trehalose is involved in the protective effect of acetic acid, and its biosynthesis is necessary to preserve plasma membrane integrity; however, the presence of trehalose in the extracellular medium also allows the cultures to maintain cell viability. Transport of trehalose was not necessary to maintain cellular viability, but was necessary to promote protection against ethanol-induced loss of plasma membrane integrity. Measurement of trehalose transport (using radioactive trehalose) and accumulation (i.e. by chromatography) in different strains and cultures media, such as, in YPD, SC and SC suplemented with trehalose in *Wt*, *tps1*Δ, *agt1*Δ strains will allow to confirm the relevance of both extra- and intracellular trehalose. Repetition of our experiments on cell viability and plasma membrane integrity of cells without capacity to synthesise and transport trehalose (*tps1*Δ*agt1*Δ*)* may add additional evidence on the dependence of acetic acid protective effect on the presence of trehalose.

Since trehalose is an osmostress protector, it will be also interesting to study the role of other osmostress protectors such as glycerol in this protective effect.

It has been described that the *hsp12*Δ strain display increased levels of intracellular trehalose in cultures stressed by freezing (Pacheco, *et al*., 2009). In this work, *hsp12*Δ cells were more resistant to ethanol than Wt cells, which could be explained by a similar increased accumulation of intracellular trehalose. The protective effect of acetic acid against ethanol-induced cell death but not ethanol-induced loss of plasma membrane integrity was still present in *hsp12*Δ cultures. Similar results were obtained with *tps1*Δ and *agt1*Δ cultures. These results reinforce that both extracellular and intracellular trehalose promotes protection by acetic acid against ethanol-induced death. Indeed trehalose transport and biosynthesis seem necessary for the maintenance of plasma membrane integrity under ethanol-induced cell death conditions. Further studies are needed to understand which pathways are induced by trehalose and its involvement in the protection against ethanol stress. Moreover previous studies demonstrated that during ethanol stress the trehalose and HSP genes are co-induced (Alexandre, *et al*., 2001; Winkler, *et al*., 1991) and a model describing this interplay has been put forward (Singer and Lindquist, 1998). It will be also interesting to analyze the cell viability and plasma membrane integrity in mutant cells on HSP genes, namely in the *hsp12*Δ mutant, in culture medium without trehalose to compare with the results we obtained in medium with trehalose (YPD).

Cell cultures without functional mitochondria did not display protection by acetic acid against ethanol-induced cell death, but still against loss of plasma membrane integrity. We can therefore conclude that mitochondrial functions, namely respiration, are important for acetic acid to increase cell viability of cells exposed to ethanol. Additional experiments using concentrations of ethanol above 13% (v/v) and for a longer period of time will provide a better understanding of the role of mitochondria in the protective effect of acetic acid.

Cells deficient in sfl1p showed that this yeast suppressor gene for flocculation seems not involved in this protective effect of acetic acid against ethanol stress. However, *sfl1*Δ cultures present high resistance to ethanol stress, and after 60 min more than 50% of cells incubated with 18% (v/v) of ethanol are still viable. It will be interesting to further characterize ethanol resistance in this mutant which can be very important for applied to biotechnological processes such as bioethanol productions and alcoholic beverages.

In conclusion our results indicate that trehalose, Hsp12p, Slt2/MPK1 and functional mitochondria play a role in the protection by the undissociated form of acetic acid against ethanol induced cytotoxicity.

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